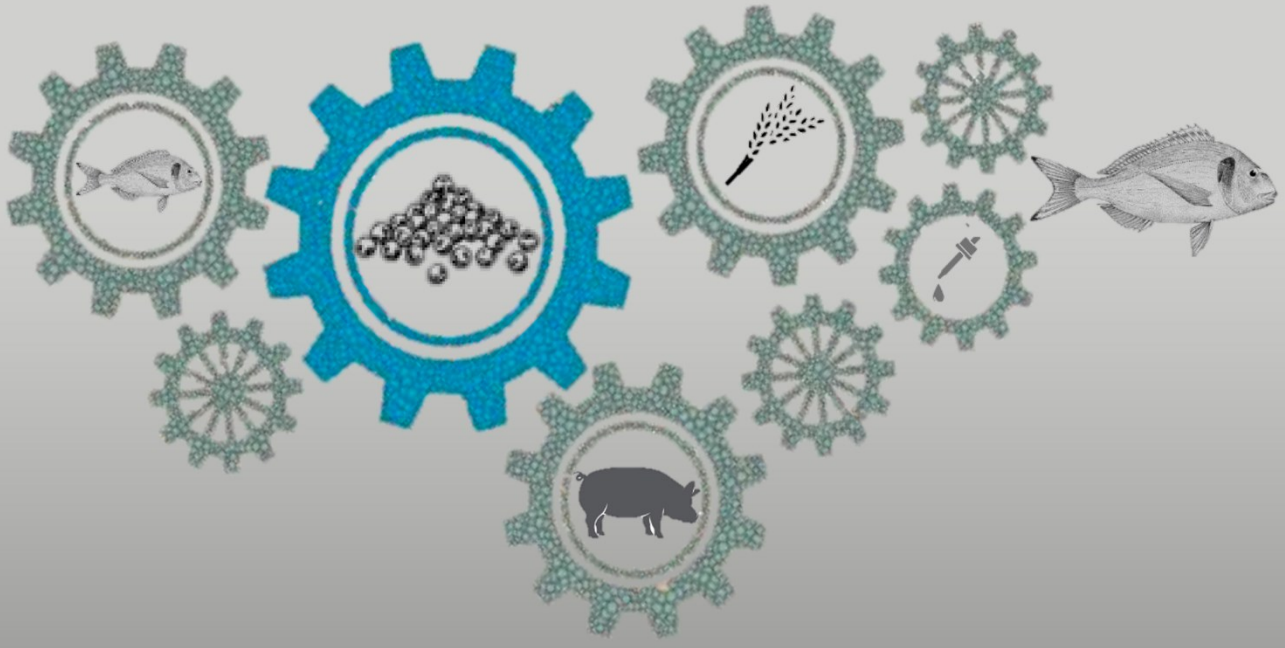
UNIVERSITAT POLITÈCNICA DE VALÈNCIA

OPTIMIZACIÓN DEL CRECIMIENTO DE PECES CARNÍVOROS ALIMENTADOS CON PIENSOS CON ALTA SUSTITUCIÓN DE HARINA DE PESCADO

Glenda María Vélez Calabria

Tesis Doctoral en Ciencia y Tecnología de la Producción Animal



Directoras:
Dra. Silvia Martínez Llorens
Dra. Ana Tomás Vidal

Junio de 2024

UNIVERSIDAD POLITÉCNICA DE VALENCIA

INSTITUTO DE CIENCIA Y TECNOLOGÍA ANIMAL

GRUPO DE ACUICULTURA Y BIODIVERSIDAD

Optimización del crecimiento de peces carnívoros alimentados con piensos con alta sustitución de harina de pescado

Growth optimization of carnivorous fish fed with feeds with high fishmeal substitution

"Nuestra recompensa se encuentra en el esfuerzo y no en el resultado. Un esfuerzo total es una victoria completa"

Mahatma Gandhi

Dedicado a ti, Ninfa Calabria

En primer lugar, agradezco infinitamente a Dios por ayudarme a culminar este proyecto, por darme el valor y la fuerza necesaria para hacer este sueño realidad.

Doy gracias también a mi familia, por brindarme su apoyo y amor durante todo este tiempo. Gracias papá por enseñarme desde pequeña a hacer lo correcto, por los valores y principios inculcados; gracias a mi hermana, por ser pieza fundamental en mi vida, por ser quien me apoya en mis momentos de crisis existencial y desfallecimiento, quien me impulsa a continuar y a no rendirme, quien me motiva a ser mejor cada día; gracias a mi hermano, mi cuñada y mis amados sobrinos por su cariño y respaldo.

Agradezco a todo el grupo de Acuicultura por la confianza depositada en mí, por recibirme con los brazos abiertos y darme la oportunidad de formar parte de este maravilloso equipo. Gracias a Miguel Jover, a David Sánchez y especialmente a mis directoras Ana Tomás y Silvia Martínez, por su invaluable ayuda, consejos, paciencia y todo el tiempo dedicado a mí y a este proyecto.

Gracias a mis compañeros y personal del laboratorio, a Andrés Moñino por ser tan bella persona y buen amigo; a Javier Moya, por todo su apoyo, colaboración y palabras de aliento; a Sergio Godoy, Guillem Estruch, Eslam Tefal por su valiosa ayuda; a Mari, Nacho y Javi, por su amistad y complicidad, y a ti querida Raquelita, gracias por ser mi ángel guardián que no me desampara.

A Eberhard, mi maestro y amigo, a quien considero mi segundo padre, gracias por ser mi modelo a seguir, por ser mi guía durante mi vida académica y profesional, por mostrarme que la constancia y la disciplina son indispensables para alcanzar tus metas; a Gloria, Pedro y Socorrito, gracias por su amistad sincera, por ser mis consejeros de vida, por su acompañamiento en tiempos difíciles y por todos los gratos momentos compartidos.

A Yorce, Fania Alicia, Diana, Jhon Mario, Adriana, Judith e Indira, gracias por su cariño y por demostrarme que la distancia no es impedimento para una verdadera amistad; Rebeca y Andreina, gracias por estar presente en los buenos y malos momentos, por su tolerancia, cuidados, afecto y ese lazo de hermandad que hemos construido; muchas gracias a todos aquellos que me acompañaron y apoyaron a lo largo de este proceso.

NOTA PREVIA

La presente tesis doctoral está estructurada en una introducción general, justificación y objetivos, un compendio de artículos, una discusión general y las conclusiones. Todos los coautores no doctores han expresado su aceptación para la inclusión de los artículos científicos en esta tesis y su renuncia a su presentación como parte de otra tesis doctoral.

A continuación, se relacionan los artículos científicos publicados en revistas indexadas:

Vélez-Calabria, G., Martínez-Llorens, S., Milián-Sorribes, M. C., Jauralde, I., Jover-Cerdá, M., Tomás-Vidal, A. (2021). Fishmeal substitution by Iberian pig meal and vegetable proteins blend and inclusion of *Isochrysis* aff. *galbana* (T-Iso) in diets for gilthead seabream (*Sparus aurata* L.): Effects on growth and feed utilization efficiency. *Aquaculture Nutrition, 27*, 2169– 2181. https://doi.org/10.1111/anu.13352

Vélez-Calabria, G.; Peñaranda, D.S.; Jover-Cerdá, M.; Martínez-Llorens, S.; Tomás-Vidal, A. (2021). Successful inclusion of high vegetable protein sources in feed for rainbow trout without decrement in intestinal health. *Animals*, *11*, 3577. https://doi.org/10.3390/ani11123577

Vélez-Calabria, G.; Tomás-Vidal, A.; Peñaranda, D.S.; Jover-Cerdá, M.; Martínez-Llorens, S. (2023). Effect of additives inclusion in gilthead seabream (*Sparus aurata* L.) diets on growth, enzyme activity, digestibility and gut histology fed with vegetable meals. *Animals*, *13*, 205. https://doi.org/10.3390/ani13020205

Por otra parte, los trabajos realizados también fueron difundidos a través de las siguientes comunicaciones en formato póster en congresos:

Efecto de la sustitución total de harina de pescado por una mezcla de proteína vegetal en el crecimiento y parámetros nutritivos de la trucha arco iris (*Oncorhynchus mykiss* W.). Vélez-Calabria, G., Tefal, E., Estruch, G., Godoy, S., Milián-Sorribes, María C., Monge, R., Martínez-Llorens, S., Moñino, A.V., Jauralde, I., Jover-Cerdá, M. & Tomás-Vidal, A. XVI Congreso Nacional de Acuicultura. Zaragoza, España. 3 al 5 de octubre de 2017.

Partial and total replacement of fishmeal by a vegetable and animal proteins mixture and inclusion of *Isochrysis galbana* in diets for gilthead seabream (*Sparus aurata* L.). Vélez-Calabria, G., Martínez-Llorens, S., Milián-Sorribes, María C., Estruch, G., Monge, R., Godoy, S., Moñino, A.V., Jover-Cerdá, M. & Tomás-Vidal, A. Aquaculture Europe 2017 - Cooperation for Growth. Dubrovnik, Croatia. October 17-20, 2017.

RESUMEN

En la presente tesis doctoral, se llevaron a cabo diferentes experimentos en dorada (*Sparus aurata* Linnaeus, 1758) y trucha arco iris (*Oncorhynchus mykiss* Walbaum, 1792), como especies representantes de la producción acuícola marina y continental, respectivamente, con el objetivo contribuir a la optimización de los piensos acuícolas con alta y total sustitución de la harina de pescado (HP), así como la inclusión de aditivos evaluando el crecimiento, la eficiencia nutritiva y la salud intestinal de los peces. Además, se realizó un análisis del ciclo de vida para evaluar la sostenibilidad de las diferentes formulaciones de piensos, como parte de la discusión general de la tesis.

El primer experimento se llevó a cabo con truchas arco iris de 13 g de peso medio inicial y finalizó tras 11 semanas. Los animales fueron alimentados con piensos con tres niveles de sustitución de la HP (100%, 90% y 80%, denominadas FM0, FM10 y FM20, respectivamente) por fuentes de proteínas vegetales (gluten de trigo y torta de soja), considerándose la dieta FM20 como dieta control. Se evaluó el crecimiento, la mortalidad y la salud intestinal de la trucha arco iris. Se encontraron diferencias significativas en el crecimiento, con un menor peso final y TCI para los peces alimentados con la dieta FM0, al igual que en la supervivencia. La TAD y el ICA también variaron de acuerdo al tratamiento, siendo mayor para la dieta FM0 y en consecuencia registrando el menor CEC. A nivel histológico, el grupo FM0 registró los valores más altos de AV, espesor de la lámina propia (LP) y el valor más bajo de CC tanto en el intestino proximal (IP) como en el intestino distal (ID). En el análisis de la expresión génica, se estudiaron los genes *Interleucina 1β* (*IL-1β*), *Interleucina 8* (*IL-8*) y *Factor de Crecimiento Transformante beta* (*TGF-β*), genes inflamatorios relacionados con la inflamación primaria, y los genes *Transferrina*, *Inmunoglobulina T* (*IgT*) e *Interferón gamma* (*IFN-γ*), genes inmunes relacionados con la respuesta posterior del sistema inmunológico. Los peces alimentados con la dieta FM0 presentaron una mayor expresión tanto en los genes inmunes como inflamatorios, con altas expresiones de los genes *IFN-γ*, *IL-8* e *IL-1β*. Estos resultados indican que el 90% de la sustitución de HP en piensos para esta especie es posible cuando se usa una mezcla de proteínas vegetales.

En un segundo experimento, se evaluó el uso de dos aditivos (mucosa porcina hidrolizada-MPH y un concentrado de nucleótidos-NT) como suplementos en piensos

para doradas con sustitución total de la HP (100% proteína vegetal), con el objetivo de determinar los efectos sobre el crecimiento, eficiencia alimenticia, digestibilidad de la proteína e histología intestinal. El experimento se inició con doradas de 11 g de peso medio y se finalizó tras 134 días alcanzando los peces un peso de 100 g aproximadamente. Se formularon seis dietas, FM100 y AA0 como dietas control (proteína basada 100% en HP y harinas vegetales, respectivamente), P1 y P2 (1% y 2% de MPH), N250 y N500 (250 mg/kg y 500 mg/kg de NT) con diferentes niveles de inclusión de aditivos. Todos los grupos mostraron una mejora significativa en el peso corporal final y la tasa de crecimiento instantáneo (TCI) en comparación con el grupo control (AA0, sin harina de pescado ni aditivos), que presentó los valores más bajos, pero sin diferencias en la supervivencia (S). No se detectaron diferencias estadísticas significativas entre los grupos para la tasa de alimentación diaria (TAD) aunque el índice de conversión del alimento (ICA) fue significativamente mejor cuando los aditivos se agregaron en porcentajes más bajos (P1 y N250), sin diferencias significativas en comparación con la dieta FM100. De manera similar, el coeficiente de eficacia de crecimiento (CEC) presentó valores iguales y/o cercanos a la dieta FM100, cuando los aditivos se incluyeron en niveles más bajos. No se encontraron diferencias significativas en los índices biométricos, a excepción del índice hepatosomático (IHS), que en el grupo N500 fue el de menor valor. Los resultados obtenidos en los coeficientes de digestibilidad aparente (CDA) fueron similares en todos los tratamientos. A nivel histológico se observaron diferencias significativas tanto en la anchura de las vellosidades (AV) como en el recuento de células caliciformes (CC). Las actividades enzimáticas digestivas presentaron diferencias significativas, con excepción de la pepsina y la α-amilasa. Los peces alimentados con la dieta AA0 tuvieron los valores de actividad de enzimas digestivas más bajos, salvo la α-amilasa. Los resultados señalaron que, tanto la suplementación con MPH, así como con NT, puede mejorar el crecimiento de los peces y que su inclusión en bajas dosis, mejora los parámetros nutritivos y la capacidad digestiva.

Un tercer experimento evaluó el efecto de la sustitución parcial y total de HP en dietas para doradas. La dieta control (FM100) contenía HP como principal fuente proteica, en las dietas FM25, FM10 y FM0 se sustituyó la HP en un 75%, 90% y 100% por una mezcla de harinas proteicas vegetales y animal (cerdo ibérico), y en la dieta FM0+ (misma formulación que FM0) se incluyeron 50 g/kg de la microalga *Isochrysis* aff. *galbana* (T-Iso) como aditivo. El experimento se inició con peces de 64 g de peso medio. Tras 88

días de experimento, los peces alimentados con los piensos FM25 y FM100 (dieta control) presentaron el mejor crecimiento y los peces alimentados con las dietas FM0+ y FM10 alcanzaron un mayor peso y TCI que los alimentados con la dieta FM0. No se presentaron diferencias significativas en los índices biométricos, salvo en el FC, cuyo valor fue mayor en el grupo FM0+ respecto a FM0, similar a FM10 y FM25, pero inferior a FM100. El valor productivo de la proteína (VPP) y el valor productivo de la energía (VPE) fueron más bajos en los peces alimentados con la dieta FM0, mientras que el grupo FM100 presentó las mayores eficiencias. Con estos resultados se pudo concluir que hasta un 75% de sustitución de HP por una mezcla de proteínas vegetales y animal en dietas para dorada es factible para un óptimo crecimiento. La inclusión de *Isochrysis* aff. *galbana* (T-Iso) a los niveles ensayados mejoró las eficiencias de crecimiento y retención proteica y energética en una dieta sin HP.

En conclusión, en este trabajo se ha comprobado que es factible realizar altas sustituciones de harina de pescado por fuentes proteicas vegetales y animales, sin afectar el crecimiento y la salud de los peces. Del mismo modo, la inclusión de aditivos en los piensos mejora el crecimiento, la eficiencia de utilización del alimento y el estado sanitario de los peces. Las dietas con un 100% de proteína vegetal y nucleótidos presentaron un excelente beneficio económico, lo que proporcionaría un mejor crecimiento económico de la industria acuícola dado el actual incremento de costes de los alimentos para peces. Además, la sustitución total de harina de pescado y la inclusión de aditivos redujo notablemente los índices FIFO, brindando grandes posibilidades de tener una acuicultura cada vez más sostenible. Las evaluaciones del análisis del ciclo de vida indicaron que la harina de cerdo ibérico y la microalga *I.* aff. *galbana* (T-Iso) generan el mayor impacto ambiental.

ABSTRACT

In the present doctoral thesis, different experiments were carried out on gilthead seabream (*Sparus aurata* Linnaeus, 1758) and rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792), as representative species of marine and continental aquaculture production, respectively, to contribute to the optimization of aquafeeds with high and total fishmeal (FM) replacement, as well as the additives inclusion evaluating the growth, nutritional efficiency and intestinal health of fish. In addition, a life cycle assessment was performed to assess the sustainability of different feed formulations, as part of the general discussion of the thesis.

The first experiment was carried out with rainbow trout of 13 g mean initial weight and ended after 11 weeks. The animals were fed feeds with three levels of FM replacement (100%, 90% and 80%, called FM0, FM10 and FM20, respectively) by plant protein sources (wheat gluten and soybean meal), with the FM20 diet being considered as the control diet. Growth, mortality, and intestinal health of rainbow trout were evaluated. Significant differences were found in growth, with a lower final weight and SGR for the fish fed with the FM0 diet, as well as in survival. The feed intake (FI) and the feed conversion ratio (FCR) also varied according to the treatment, being higher for the FM0 diet and consequently registering the lowest protein efficiency ratio (PER). At the histological level, the FM0 group recorded the highest values of VT, lamina propria thickness (LP) and the lowest value of GC in both the proximal intestine (PI) and distal intestine (DI). In the gene expression analysis, the genes *Interleukin 1β* (*IL-1β*), *Interleukin 8* (*IL-8*), *Transforming Growth Factor beta* (*TGF-β*), inflammatory genes related to primary inflammation, and the genes *Transferrin*, *Immunoglobulin T* (*IgT*) and *Interferon gamma* (*IFN-γ*), immune genes related to the subsequent response of the immune system, were studied. Fish fed the FM0 diet had higher expression of both immune and inflammatory genes, with high expressions of the *IFN-γ*, *IL-8* and *IL-1β* genes. These results indicate that 90% of FM replacement in feed for this species is possible when a plant protein blend is used.

In a second experiment, the use of two additives (hydrolyzed porcine mucosa-HPM and a nucleotide concentrate-NT) as supplements in feeds for gilthead seabream with total FM replacement (100% plant protein) was evaluated, intending to determine the possible

effects on growth, feed efficiency, protein digestion and gut histology. The experiment started with gilthead seabream of 11 g average weight and ended with fish of approximately 100 g after 134 days. Six diets were formulated, FM100 and AA0 as control diets (100% FM-based protein and vegetable meals, respectively), P1 and P2 (1% and 2% HPM), N250 and N500 (250 mg/kg and 500 mg/kg NT) with different levels of additive inclusion. All groups showed a significant improvement in final body weight and specific growth rate (SGR) in comparison with the control group (AA0, non-fishmeal or additives), which had the lowest values, but no difference in survival (S). No significant statistical differences were detected between groups for feed intake (FI), although feed conversion ratio (FCR) was significantly better when additives were added at lower percentages (P1 and N250), with no significant differences compared to the FM100 diet. Similarly, the protein efficiency ratio (PER) presented values equal to and/or close to the FM100 diet when the additives were included in lower levels. No significant differences were found in the biometric indices, with the exception of the hepatosomatic index (HSI), which in the N500 group was the lowest value. The apparent digestibility coefficients (ADC) results were similar in all treatments. Significant differences were observed at the histological level in villi thickness (VT) and goblet cells (GC). Digestive enzyme activities showed significant differences, with the exception of pepsin and α-amylase. Fish fed the AA0 diet had the lowest digestive enzyme activity values, except for α-amylase. The results indicated that HPM and NT supplementation can improve fish growth and that their inclusion in low doses improves nutritional parameters and digestive capacity.

The effect of partial and total FM replacement in gilthead seabream diets was evaluated in a third experiment. The control diet (FM100) contained FM as the main protein source, in the FM25, FM10 and FM0 diets, 75%, 90% and 100% of the FM was replaced by a plant and animal (Iberian pig) proteins blend, and in the FM0+ diet (same formulation as FM0) 50 g/kg of the microalgae *Isochrysis* aff. *galbana* (T-Iso) was included as an additive. The experiment was started with fish of 64 g average weight. After 88 days of the experiment, the fish fed with the FM25 and FM100 diets showed the best growth and the fish fed with FM0+ and FM10 diets reached a higher weight and SGR than those fed FM0 diet. There were no significant differences in the biometric indices, except for CF, whose value was higher in the FM0+ group compared to FM0, similar to FM10 and FM25, but lower than FM100. The retention efficiency of protein (PIR) and energy intake

(EIR) were lowest in the fish fed with the FM0 diet, while the FM100 group had the highest efficiencies. With these results, it was possible to conclude that up to 75% FM replacement by a plant and animal proteins blend in diets for gilthead seabream is feasible for optimal growth. The inclusion of *Isochrysis* aff. *galbana* (T-Iso) at the levels tested improved growth efficiencies and protein and energy retention in a fishmeal-free diet.

In conclusion, in this work it has been proven that it is feasible to make high replacement of FM by plant and animal protein sources, without affecting the growth and health of the fish. Similarly, the inclusion of additives in feeds improves growth, feed utilization efficiency and fish health status. Diets with 100% plant protein and nucleotides presented an excellent economic benefit, which would provide better economic growth for the aquaculture industry given the current increase in fish feed costs. In addition, the total FM replacement and the inclusion of additives significantly reduced FIFO indices, providing great possibilities for increasingly sustainable aquaculture. Life cycle assessment evaluations indicated that Iberian pig meal and the microalgae *I.* aff. *galbana* (T-Iso) generate the greatest environmental impact.

RESUM

En la present tesi doctoral, es van dur a terme diferents experiments en orades (*Sparus aurata* Linnaeus, 1758) i truites arc iris (*Oncorhynchus mykiss* Walbaum, 1792) com a espècies representants de la producció aqüícola marina i continental, respectivament, amb l'objectiu contribuir a l'optimització dels pinsos aqüícoles amb alta i total substitució de la farina de peix (FP), així com la inclusió d'additius avaluant el creixement, l'eficiència nutritiva i la salut intestinal dels peixos. A més, es va realitzar una anàlisi del cicle de vida per avaluar la sostenibilitat de les diferents formulacions de pinsos, com a part de la discussió general de la tesi.

El primer experiment es va dur a terme amb truites arc iris de 13 g de pes mitjà inicial i va finalitzar després d'11 setmanes. Els animals van ser alimentats amb pinsos amb tres nivells de substitució de la FP (100%, 90% i 80%, denominades FM0, FM10 i FM20, respectivament) per fonts de proteïnes vegetals (gluten de blat i tortó de soja), considerant-se la dieta FM20 com a dieta control. Es va avaluar el creixement, la mortalitat i la salut intestinal de la truita arc iris. Es van trobar diferències significatives en el creixement, amb un menor pes final i TCI per als peixos alimentats amb la dieta FM0, igual que en la supervivència. La TAD i el ICA també van variar d'acord amb el tractament, sent major per a la dieta FM0 i en conseqüència registrant el menor CEC. A nivell histològic, el grup FM0 va registrar els valors més alts de AV, grossària de la làmina pròpia (LP) i el valor més baix de CC tant en l'intestí proximal (IP) com en l'intestí distal (ID). En l'anàlisi de l'expressió gènica, es van estudiar els gens *Interleucina 1β* (*IL-1β*), *Interleucina 8* (*IL-8*) i *Factor de Crecimiento Transformante beta* (*TGF-β*), gens inflamatoris relacionats amb la inflamació primària, i els gens *Transferrina*, *Inmunoglobulina T* (*IgT*) e *Interferón gamma* (*IFN-γ*), gens immunes relacionats amb la resposta posterior del sistema immunològic. Els peixos alimentats amb la dieta FM0 van presentar una major expressió tant en els gens immunes com inflamatoris, amb altes expressions dels gens *IFN-γ*, *IL-8* i *IL-1β*. Aquests resultats indiquen que el 90% de la substitució de FP en pinsos per a aquesta espècie és possible quan s'utilitza una mescla de proteïnes vegetals.

En un segon experiment, es va avaluar l'ús de dos additius (mucosa porcina hidrolitzada-MPH i un concentrat de nucleòtids-NT) com a suplements en pinsos per a ourades amb

substitució total de la FP (100% proteïna vegetal), amb l'objectiu de determinar els efectes sobre el creixement, eficiència alimentosa, digestibilitat proteica i histologia intestinal. L'experiment es va iniciar amb orades d'11 g de pes mitjà i es va finalitzar després de 134 dies aconseguint els peixos un pes de 100 g aproximadament. Es van formular sis dietes, FM100 i AA0 com a dietes control (proteïna basada 100% en FP i farines vegetals, respectivament), P1 i P2 (1% i 2% de MPH), N250 i N500 (250 mg/kg i 500 mg/ kg de NT) amb diferents nivells d'inclusió d'additius. Tots els grups van mostrar una millora significativa en el pes corporal final i la taxa de creixement instantani (TCI) en comparació del grup control (AA0, sense farina de peix ni additius), que va presentar els valors més baixos, però sense diferències en la supervivència (S). No es van detectar diferències estadístiques significatives entre els grups per a la taxa d'alimentació diària (TAD) encara que l'índex de conversió de l'aliment (ICA) va ser significativament millor quan els additius es van agregar en percentatges més baixos (P1 i N250), sense diferències significatives a comparació amb la dieta FM100. De manera similar, el coeficient d'eficàcia de creixement (CEC) va presentar valors iguals i/o pròxims a la dieta FM100, quan els additius es van incloure en nivells més baixos. No es van trobar diferències significatives en els índexs biomètrics, a excepció de l'índex hepatosomàtic (IHS), que en el grup N500 va ser el de menor valor. Els resultats obtinguts en els coeficients de digestibilitat aparent (CDA) van ser similars en tots els tractaments. A nivell histològic es van observar diferències significatives tant en l'amplària de les vellositats (AV) com en el recompte de cèl·lules caliciformes (CC). Les activitats enzimàtiques digestives van presentar diferències significatives, amb excepció de la pepsina i la α-amilasa. Els peixos alimentats amb la dieta AA0 van tindre els valors d'activitat d'enzims digestius més baixos, tret de la α-amilasa. Els resultats van assenyalar que, tant la suplementació amb MPH, així com amb NT, pot millorar el creixement dels peixos i que la seua inclusió en baixes dosis, millora els paràmetres nutritius i la capacitat digestiva.

Un tercer experiment va avaluar l'efecte de la substitució parcial i total de FP en dietes per a orades. La dieta control (FM100) contenia FP com a principal font proteica, en les dietes FM25, FM10 i FM0 es va substituir la FP en un 75%, 90% i 100% per una mescla de farines proteiques vegetals i animal (porc ibèric), i en la dieta FM0+ (mateixa formulació que FM0) es van incloure 50 g/kg de la microalga *Isochrysis* aff. *galbana* (T-Iso) com a additiu. L'experiment es va iniciar amb peixos de 64 g de pes mitjà. Després de 88 dies d'experiment, els peixos alimentats amb els pinsos FM25 i FM100 (dieta

control) van presentar el millor creixement i els peixos alimentats amb les dietes FM0+ i FM10 van aconseguir un major pes i TCI que els alimentats amb la dieta FM0. No es van presentar diferències significatives en els índexs biomètrics, excepte en l'FC, el valor del qual va ser major en el grup FM0 + respecte a FM0, similar a FM10 i FM25, però inferior a FM100. El valor productiu de la proteïna (VPP) i el valor productiu de l'energia (VPE) van ser més baixos en els peixos alimentats amb la dieta FM0, mentre que el grup FM100 va presentar les majors eficiències. Amb aquests resultats es va poder concloure que fins a un 75% de substitució de FP per una mescla de proteïnes vegetals i animal en dietes per a orada és factible per a un òptim creixement. La inclusió de *Isochrysis* aff. *galbana* (T-Iso) als nivells assajats va millorar les eficiències de creixement i retenció proteica i energètica en una dieta sense FP.

En conclusió, en aquest treball s'ha comprovat que és factible realitzar altes substitucions de farina de peix per fonts proteiques vegetals i animals, sense afectar el creixement i la salut dels peixos. De la mateixa manera, la inclusió d'additius en els pinsos millora el creixement, l'eficiència d'utilització de l'aliment i l'estat sanitari dels peixos. Les dietes amb un 100% de proteïna vegetal i nucleòtids van presentar un excel·lent benefici econòmic, la qual cosa proporcionaria un millor creixement econòmic de la indústria aqüícola donat l'actual increment de costos dels aliments per a peixos. A més, la substitució total de farina de peix i la inclusió d'additius va reduir notablement els índexs FIFO, brindant grans possibilitats de tenir una aqüicultura cada vegada més sostenible. Les avaluacions de l'anàlisi del cicle de vida van indicar que la farina de porc ibèric i la microalga *I.* aff. *galbana* (T-Iso) generen el major impacte ambiental.

ABREVIATURAS

Castellano

HP – Harina de pescado

AP – Aceite de pescado

MPH – Mucosa porcina hidrolizada

NT – Nucleótidos

HcI – Harina de cerdo ibérico

TCI – Tasa de crecimiento instantáneo

TAD – Tasa de alimentación diaria

S – Supervivencia

ICA – Índice de conversión del alimento

CEC – Coeficiente de eficacia de crecimiento

FC – Factor de condición

IVS – Índice viscerosomático

IGV – Índice de grasa visceral

IHS – Índice hepatosomático

CDA – Coeficiente de digestibilidad aparente

VPP – Valor productivo de la proteína

VPE – Valor productivo de la energía

AV – Anchura de las vellosidades

CC – Células caliciformes

LP- Lámina propia

IP – Intestino proximal

ID – Intestino distal

IL-1β – Interleucina 1 beta

IL-8 – Interleucina 8

TGF-β – Factor de crecimiento transformante beta

IgT – Inmunoglobulina T

IFN-γ – Interferón gamma

FAN – Factores antinutricionales

UE – Unión Europea

AA – Aminoácidos

AAE – Aminoácidos esenciales

AG – Ácidos grasos

PAP – Proteínas animales procesadas

SRA – Sistemas de recirculación acuícola

PV – Proteínas vegetales

ICE – Índice de conversión económico

IBE – Índice de beneficio económico

IBEst - Índice de beneficio económico estándar

<u>English</u>

FM – Fishmeal

IPM – Iberian pig meal

HPM – Hydrolyzed porcine mucosa

NT – Nucleotide

FIFO – Fish in/Fish out

FFDR – Forage fish dependency ratio

DNA – Deoxyribonucleic acid

RNA – Ribonucleic acid

IMP – Inosine monophosphate

GMP – Guanosine monophosphate

AMP – Adenosine monophosphate

CP – Crude protein

CL – Crude lipid

CF – Crude fiber

CHO – Total carhohydrates

DM – Dry matter

NFE – nitrogen-free extract

AA – Amino acid

EAA – Essential amino acid

NEAA – Non-essential amino acid

SGR – Specific growth rate

FI – Feed intake

FCR – Feed conversion ratio

PER – Protein efficiency ratio

S – Survival

CF – Condition factor

VSI – Viscerosomatic index

VFI – Visceral fat index

HSI – Hepatosomatic index

ADC – Apparent digestibility coefficient

PI – Proximal intestine

MI – Middle intestine

DI – Distal intestine

SL – Serous layer

ML – Muscular layer

SML – Submucous layer

VL – Villi Length

VT – Villi thickness

LP – Lamina propria

GC – Goblet cells

ALP – Alkaline phosphatase

BB – Brush border

PP – Plant protein

PAP – Processed animal proteins

ANF – Antinutritional factors

IPM – Iberian pig meal

MBM – Meat and bone meal

PBM – Poultry by-product meal

SBM – Soybean meal

SPC – Soybean protein concentrate

WM – Wheat meal

WG – Wheat gluten

EPA – Eicosapentanoic acid

PUFA – Polyunsaturated fatty acids

PIR – retention efficiency of protein intake

EIR – retention efficiency of energy intake

AAIRE – retention efficiency of amino acid

PER – Protein efficiency ratio

IL-1β – Interleukin-1 beta

IL-8 – Interleukin 8

TGF-β – Transforming Growth Factor beta

IgT – Immunoglobulin T

IFN-γ – Interferon gamma

β-actin – Beta actin

ELF-1α – Elongation Factor 1-alpha

LCA – Life Cycle Assessment

GWP – Global Warming Potential

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DISCUSIÓN GENERAL

INTRODUCCIÓN GENERAL

1.1. La producción acuícola

En las últimas dos décadas, el sector acuícola, aunado al de la pesca, han sido reconocidos por su contribución fundamental a la seguridad alimentaria y la nutrición de la población mundial. En 2020, la producción mundial pesquera y acuícola alcanzó un récord histórico de 214 millones de toneladas, que comprendían 178 millones de toneladas de animales acuáticos y 36 millones de toneladas de algas. Se prevé que la producción total de la pesca y la acuicultura (excluidas las algas) siga aumentando y alcance los 202 millones de toneladas en 2030. Ello supone un incremento del 14% con respecto a 2020. La mayor parte del aumento de la producción pesquera y acuícola mundial provendrá del sector de la acuicultura, donde el volumen total debería superar el umbral de los 100 millones de toneladas por primera vez en 2027. Se prevé que la producción acuícola aumente a 106 millones de toneladas en 2030, lo que supondría un incremento global del 22%, esto es, casi 19 millones de toneladas, con respecto a 2020 (FAO, 2022). Si bien este es un buen panorama, la industria acuícola ha venido enfrentando una serie de obstáculos tales como, la crisis sanitaria causada por el COVID-19, la invasión rusa de Ucrania a inicios del año 2022 que ha logrado exacerbar la inseguridad alimentaria a nivel mundial, el aumento en el precio de materias primas vegetales para la elaboración de los piensos para peces y el alza en el precio de la energía (electricidad y combustibles), entre otros.

Actualmente, las fuentes de proteínas y lípidos alternativas a la harina de pescado (HP) y aceite de pescado (AP) se están utilizando en la acuicultura debido a los inconvenientes que éstos conllevan como la insostenibilidad, el aumento de precios, la necesidad de obtenerlos de la pesca extractiva, la oxidación lipídica de sus ácidos grasos, etc. (Gatlin III et al., 2007a; Hardy, 2010). Por muchos años, las investigaciones del sector acuícola se han centrado en desarrollar formulaciones de piensos que reduzcan la dependencia de los recursos marinos limitados. Mientras que la producción de especies de peces herbívoros y omnívoros ha pasado fácilmente a alimentos que contienen poca o ninguna HP o AP, tales formulaciones han sido más difíciles de implementar en la alimentación de peces carnívoros y crustáceos (Turchini et al., 2019). En el caso de especies de peces carnívoros, incluso la soja, la proteína vegetal más completa con relación a su precio, es problemática con respecto al sabor, los factores antinutricionales (FAN) y la capacidad de satisfacer las necesidades nutricionales diarias (Francis et al., 2001; Papatryphon and Soares, 2000). De acuerdo a lo anterior, mejorar la sostenibilidad de los alimentos

acuícolas es crucial para satisfacer la creciente demanda mundial de productos del mar y hacer de la acuicultura un sistema alimentario más sostenible que pueda contribuir a la transición ecológica, como se espera de la estrategia «de la granja a la mesa» establecida por el Pacto Verde Europeo (European Commission, 2021; FAO, 2020; Lorenzo and Simal-Gandara, 2021; Tacon et al., 2022).

La trucha arco iris (*Oncorhynchus mykiss* Walbaum, 1792) es la principal especie de agua dulce producida en la Unión Europea (UE), tanto en volumen como en valor (EUMOFA, 2021) y su producción se basa principalmente en la acuicultura, ya que las capturas suponen un porcentaje muy pequeño dentro de las capturas totales de la UE. La producción acuícola mundial de trucha arco iris en 2020 fue de 959.694 toneladas y su producción en España en 2021 se estima que fue de 15.357 toneladas (APROMAR, 2022). En los países de la UE, la trucha arco iris se produce para el consumo humano y para la repoblación de cuerpos de agua para la pesca recreativa (EUMOFA, 2021) y alrededor del 56% de los especímenes jóvenes se crían en sistemas de recirculación acuícola (SRA) (EUMOFA, 2020).

Por otro lado, la dorada (*Sparus aurata* Linnaeus, 1758) es un pez teleósteo carnívoro ampliamente distribuido en los mares Mediterráneo y Atlántico. Esta especie es una de las principales especies marinas de valor comercial en el área mediterránea, con una producción mundial anual de alrededor de 280 mil toneladas (FAO, 2022) y una producción en España (acuicultura) en 2021 de 9.632 toneladas (APROMAR, 2022).

La acuicultura se enfrenta a una serie de retos ligados a numerosos factores que influyen en la evolución y la dinámica del sector, tales como los cuellos de botella en el suministro de materias primas, las guerras, las repercusiones en los precios del petróleo, las pandemias, las sanciones comerciales, la contaminación y el cambio climático, así como los fenómenos meteorológicos extremos, que apuntan a un aumento de los costes de producción (OECD and FAO, 2022). A finales del año 2021 la reactivación económica tras la pandemia trajo consigo un notable incremento de los costes de las materias primas y servicios necesarios para el desarrollo del sector acuícola y la guerra en Ucrania ha empeorado enormemente la situación, ya que la acuicultura española ha registrado incrementos del 100% en el precio del oxígeno, la electricidad ha aumentado un 250% con respecto al 2021, el gasóleo es un 20% más costoso y el precio del pienso ha subido

de un 30% a un 50% (APROMAR, 2022). El punto más débil en la producción acuícola es el efecto de los costes de alimentación. Los precios de los piensos afectan el coste de producción en un 50% en Grecia, un 38% en España, un 22% en Italia, un 50% en Croacia, un 45% en Israel y entre un 66 y un 74% en Turquía (Özden et al., 2024). En 2021 se utilizaron en España 139.526 toneladas de pienso, un 48,6% más que el año anterior con 93.881 toneladas. La alimentación de los animales de acuicultura, es un elemento clave de su viabilidad y la optimización del uso de las materias primas, el conocimiento sobre los nutrientes, su digestibilidad y el correcto manejo del pienso son esenciales para el desarrollo responsable de esta actividad (APROMAR, 2022). Por otra parte, los precios de venta aumentaron y el valor total en primera venta de los pescados de acuicultura producidos en la UE en 2020 fue de unos 3.050,1 millones de euros, lo que supone un incremento del 2,2% respecto de 2019 (APROMAR, 2022). Se prevé que los precios de venta disminuyan en los próximos diez años, debido a la competencia cada vez mayor de otras fuentes de proteínas, predominantemente las carnes de porcino y de aves de corral (OECD and FAO, 2023). Sin embargo, el moderado crecimiento de la producción acuícola impedirá que los precios bajen demasiado (OECD and FAO, 2023).

La pandemia ha repercutido en el trabajo, los ingresos y el poder adquisitivo, agravando la falta de acceso a una alimentación adecuada que padecen millones de personas, lo cual convierte su seguridad alimentaria en un problema enorme y persistente. Aunque la pandemia, la posterior recuperación económica, la guerra en Ucrania y el aumento de los precios de los alimentos, los insumos agrícolas y la energía han tenido efectos diferentes en las distintas regiones, las nuevas estimaciones indican que el hambre ya no está aumentando a nivel mundial, pero sigue estando muy por encima de los niveles anteriores a la pandemia. A nivel mundial, en 2019, los alimentos acuáticos proporcionaron alrededor del 17% de las proteínas de origen animal y el 7% de las proteínas totales. Se prevé que, en 2030, el 90% de toda la producción de animales acuáticos se destinará al consumo humano, un incremento general del 15% en comparación con 2020. Esto significa que el consumo per cápita anual aumentará de 20,2 kg en 2020 a 21,4 kg en 2030 (FAO, 2022).

1.2. Las harinas de pescado y la sostenibilidad de la acuicultura

La sostenibilidad y el crecimiento de una acuicultura intensiva de peces carnívoros se ha visto amenazada en los últimos años por su dependencia a la HP y el AP (Cardinaletti et al., 2019), ya que éstas se han utilizado como las principales fuentes de proteína y lípidos en los alimentos acuícolas. La HP y el AP se producen principalmente de especies de peces forrajeros, es decir, especies que desempeñan un papel vital en los ecosistemas al transferir energía de los productores primarios a especies de nivel trófico superior (Cashion et al., 2017). La HP representa una fuente de proteínas de alta calidad con un perfil equilibrado de aminoácidos esenciales (AAE) y ácidos grasos (AG), alta digestibilidad y buena palatabilidad (Olsen and Hasan, 2012). La eliminación o reducción de la HP en las dietas acuícolas fue la puerta de entrada para que las investigaciones en nutrición de peces, proporcionaran beneficios económicos y ambientales al disminuir el coste de los alimentos, así como la reducción de la presión ejercida sobre las especies capturadas para la producción de HP que también sirven como recursos importantes en la red alimentaria marina (Kok et al., 2020; Tacon et al., 2011).

Se han logrado muchos avances en la sustitución parcial de HP con fuentes alternativas de proteínas en los alimentos para peces (Oliva-Teles et al., 2015). La cantidad de HP utilizada en dietas para especies carnívoras ha mostrado una clara tendencia decreciente hacia un uso más selectivo de HP como ingrediente estratégico en niveles más bajos, según la etapa del ciclo de vida y la especie de los peces (Hardy, 2010). Sin embargo, en general, el uso de HP en el sector de alimentos acuícolas ha seguido aumentando como consecuencia del crecimiento de la producción acuícola y el consumo relacionado de alimentos acuícolas (Oliva-Teles et al., 2015). Por el contrario, el porcentaje de HP y AP con origen extractivo en la composición del alimento de los peces de acuicultura, según datos sectoriales, ha ido disminuyendo en los últimos años, desde más del 50% en los años 90 a unos niveles inferiores al 25% en la actualidad. En general, esta reducción ha sido posible gracias a la economía circular y al aprovechamiento de subproductos de la industria conservera y de transformación del pescado. Para algunas especies, como la dorada, lubina o trucha, esta sustitución de harinas y aceites con origen extractivo por otros ingredientes, puede llegar hasta el 100% (SGP-MAPA, 2022).

Una alimentación sostenible a largo plazo, se basa en el empleo de materias primas cuyo origen, forma de obtención y procesado lo sean también, que garantice además el bienestar de los peces, cubriendo todos sus requerimientos biológicos y que asegure la calidad nutritiva y saludable del pescado. Los índices considerados internacionalmente para medir la sostenibilidad de la alimentación de los peces incluyen:

- FIFO (*Fish In/Fish Out*): que evalúa la cantidad de pescado silvestre que se necesita para producir 1 kg de pescado acuícola, en este caso diferenciado para peces marinos y peces continentales. El índice FIFO para los peces marinos se situó en 0,75 y en 0,93 para los peces continentales en 2020 (SGP-MAPA, 2022).
- FFDR (*Forage Fish Dependency Ratio*): se expresa como una tasa que toma en cuenta la cantidad de HP y AP en los alimentos balanceados que proviene de peces forrajeros y se calcula sobre una base específica del lugar teniendo en cuenta el índice de conversión del alimento (FCR por sus siglas en inglés), que permite medir la cantidad total de pienso utilizado en una granja para producir 1 kilo de pescado acuícola (IFFO, 2017). El índice FFDR en 2020 para los peces marinos fue de 0,52 y para peces continentales fue de 0,64 (IFFO, 2020).

Según Gatlin et al. (2007) la sostenibilidad económica y ambiental de la acuicultura depende de la identificación y aplicación de materias primas alternativas a la HP, con nutrientes altamente digestibles que mejoren el rendimiento de los peces, menor producción de residuos, disponibles en el mercado en grandes cantidades regulares y a un precio competitivo. Los productos que mejoran la eficiencia de los alimentos son particularmente importantes ya que los costes de los alimentos son un gasto importante en la producción acuícola (50–60% del coste total). En las dietas actuales utilizadas en acuicultura, se han empleado fuentes de proteínas alternativas a la HP, aunque algunas de ellas, como las proteínas vegetales derivadas de cereales o legumbres presentan FAN, fibra, hidratos de carbono insolubles, desequilibrios de AA y baja palatabilidad que limitan su uso y aumentan los desechos producidos en las piscigranjas aumentando el impacto ambiental de la acuicultura (Muzquiz and Wood, 2007; Naylor et al., 2009), los cuales también pueden tener efectos secundarios adversos en el crecimiento, reproducción, morfología intestinal, microbiota intestinal, estado inmunitario y salud de los peces (Colombo, 2020). A pesar de ello, se han obtenido muy buenos resultados, en cuanto al crecimiento se refiere, en truchas (Gomes et al., 1995; Lee et al., 2010;

Santigosa et al., 2008) y en doradas (Martínez-Llorens et al., 2009; Sánchez-Lozano et al., 2011), cuando la sustitución de la HP se hace con mezclas de ingredientes de proteínas vegetales. Por otro lado, los subproductos de animales terrestres tienen una mejor composición nutricional y están disponibles a bajo coste en los mercados (Naylor et al., 2009). La calidad de las proteínas en las harinas de subproductos animales variará según el origen de las materias primas y además, éstas contienen una cantidad razonable de fósforo, un nutriente importante para los animales acuáticos (Tangendjaja, 2015). Aun así, los consumidores parecen reacios a comer animales alimentados con harinas de sangre, huesos o plumas debido a las varias crisis alimentarias presentadas anteriormente y las severas restricciones posteriores sobre su uso en la alimentación animal (Jędrejek et al., 2016). En 2013, se levantó parcialmente dichas restricciones autorizando el uso de proteínas animales procesadas (PAP) derivadas de animales no rumiantes (aves y cerdos) para la alimentación de animales de acuicultura (Reglamento (UE) n.º 56/2013 de la Comisión, de 16 de enero de 2013). A la fecha, son varios los estudios que se han realizado con fuentes de proteína animal en dietas para doradas (Davies et al., 2009; Fontinha et al., 2021; Laporte, 2007; Moutinho et al., 2017; Nengas et al., 1999; Psofakis et al., 2020; Randazzo et al., 2021a; Sabbagh et al., 2019) y truchas (Bureau et al., 2000, 1999; Gaudioso et al., 2021; Lee et al., 2001; Sugiura et al., 2000).

Actualmente, una variedad de fuentes de proteínas alternativas se utiliza para la sustitución de HP en alimentos acuícolas con diferentes tasas de inclusión. La Tabla 1 presenta un resumen de los trabajos más recientes en truchas y doradas, donde se puede apreciar que la inclusión óptima de proteínas de origen vegetal puede llegar al 90%, levaduras 30%, macroalgas 10%, microalgas 75%, harinas de insectos 45% y subproductos animales 37,5%.

Tabla 1. Resumen de investigaciones recientes sobre la utilización de fuentes proteicas alternativas en trucha y dorada.

Fuente proteica alternativa	Trucha		Dorada	
	Porcentaje inclusión (%)	Autores	Porcentaje inclusión (%)	Autores
Origen vegetal	50	(Alami-Durante et al., 2010)	60	(Sánchez-Lozano et al., 2009)
	82	(Burr et al., 2012)	34	(Martínez-Llorens et al., 2012a)

	10	(Tomás-Almenar et al., 2020)	40	(Kokou et al., 2012)
	10–50	(Zhao et al., 2021)		
Levaduras	20–30	(Nazzaro et al., 2021)	0,55–1,1	(Reyes-Becerril et al., 2017)
	15	(Richard et al., 2021)	30	(Estévez et al., 2021)
	4,7	(Kheirabadi et al., 2022)	20–30	(Nazzaro et al., 2021)
	20	(Warwas et al., 2023)		
Macroalgas	1–2	(Ferreira et al., 2020)	5	(Passos et al., 2021)
	< 6	(Quiñones et al., 2021)	2,5	(Silva-Brito et al., 2022)
	5–10	(Vazirzadeh et al., 2022)	2	(Estévez and Vasilaki, 2023)
Microalgas	9	(Serrano et al., 2021)	10	(Pereira et al., 2020)
	0,36–0,50	(Arteaga Quico et al., 2021)	10	(Galafat et al., 2022)
	5–10	(Chen et al., 2022)	< 5	(Sáez et al., 2022)
	25–75	(Liu et al., 2022)	30	(Karapanagiotidis et al., 2022)
Harinas de insectos	45	(Randazzo et al., 2021b)	8,1	(Randazzo et al., 2021a)
	8	(Hossain et al., 2021)	19,5	(Mastoraki et al., 2022)
	18	(Melenchón et al., 2022)		
Subproductos animales	5	(Fehringer et al., 2014)	10,8–21,6	(Psofakis et al., 2020)
	17,8–36	(Randazzo et al., 2021b)	37,5	(Fontinha et al., 2021)
	17,8–36	(Cardinaletti et al., 2022)	5	(Fernández-Alacid et al., 2022)

1.3. Problemas asociados a la sustitución de harinas de pescado por ingredientes alternativos

El gran obstáculo común que enfrenta la industria acuícola con respecto al uso de ingredientes alternativos, es el cambio en el estado inmunológico de los peces por la introducción de una serie de FAN, especialmente en muchos subproductos vegetales (Sitjà-Bobadilla et al., 2005). No solo el estado inmunológico puede verse alterado por la sustitución de la HP, sino también la absorción intestinal puede verse afectada por estas sustituciones (Santigosa et al., 2011), a la vez que afecta directamente el crecimiento de los peces, ya que los altos niveles de sustitución de HP a menudo resultan en depresiones significativas en el rendimiento de los peces (Gatlin III et al., 2007b; Montoya-Camacho et al., 2019), conduciendo en última instancia a una supresión inmunológica, enfermedad y muerte (Figura 1).

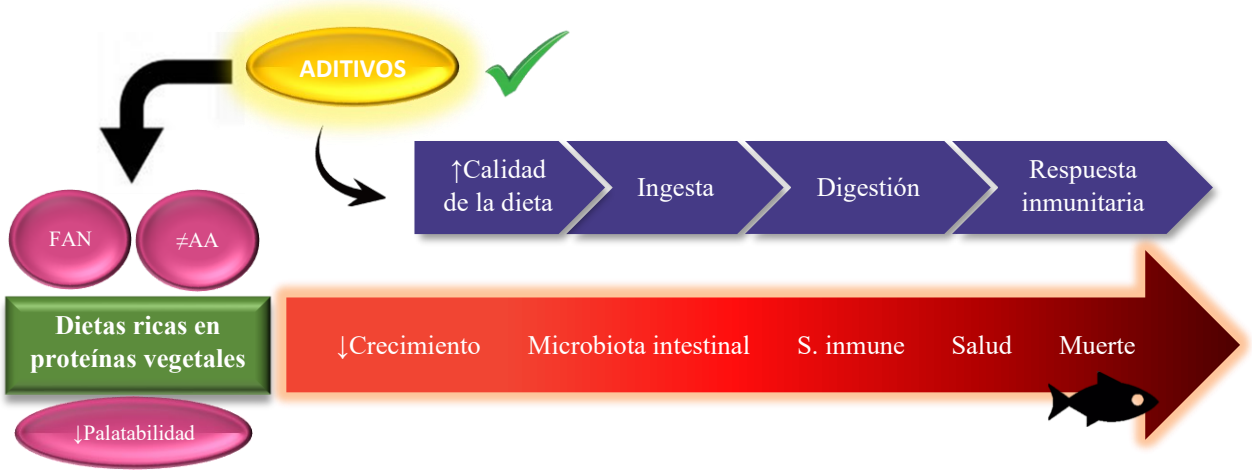

Figura 1. Modelo gráfico de la respuesta de los peces a dietas ricas en proteínas vegetales (elaboración propia).

1.4. Uso de aditivos alimentarios en piensos para peces

Como consecuencia a los problemas asociados a la sustitución de HP por ingredientes alternativos, se están buscando soluciones para resolver esta situación y una de ellas es el uso de varios aditivos alimentarios. Los aditivos alimentarios se agregan durante la preparación del alimento para mejorar la calidad del alimento, el rendimiento de la salud y la eficiencia alimentaria de los peces. La mayoría de los aditivos para alimentos no son nutritivos e incluyen antioxidantes, inmunoestimulantes, probióticos y antibióticos que se agregan para mejorar el crecimiento y la calidad del agua. Estos componentes en la alimentación acuícola aumentan el coste de producción, por lo que las empresas de

alimentos balanceados han recurrido a la aplicación de aditivos funcionales. Los aditivos alimentarios funcionales mejoran el crecimiento, la respuesta inmunitaria, induce las funciones fisiológicas y el rendimiento de la salud de los peces sobre los aditivos alimentarios normales (Bharathi et al., 2019). Los aditivos alimentarios funcionales utilizados en la acuicultura tienen una amplia gama de diversidad. Los aglutinantes, estabilizadores, antioxidantes y agentes antimicrobianos que se utilizan para preservar las características nutricionales de la dieta o los ingredientes del alimento antes de la alimentación; los estimulantes y atrayentes de alimentos (p. ej., betaína, nucleótidos, etc.) se utilizan para mejorar el consumo de alimentos y la palatabilidad de las dietas; los colorantes como la astaxantina y las xantofilas podrían ayudar con la pigmentación del producto final; las enzimas (p. ej., fitato, amilasa, etc.) y los ácidos orgánicos (p. ej., butírico) pueden facilitar el proceso de digestión y aumentar la disponibilidad de nutrientes, dando como resultado un mejor crecimiento; los inmunoestimuladores como los probióticos, prebióticos y fitogénicos se utilizan principalmente para mejorar las respuestas inmunitarias de los peces o la calidad del agua de la piscifactoría (Encarnação, 2016; NRC, 2011).

Entre los aditivos alimentarios encontramos los nucleótidos, que son compuestos intracelulares de bajo peso molecular que desempeñan un papel clave en diversas funciones fisiológicas y bioquímicas esenciales, incluida la codificación de información genética y la mediación del metabolismo energético y la transducción de señales (Carver, 1994; Carver and Allan Walker, 1995). Aunque los esfuerzos iniciales en la evaluación de la suplementación dietética de nucleótidos para peces se remontan a principios de la década de 1970, la investigación en ese momento se centró principalmente en los posibles efectos quimioatrayentes de estos compuestos (Kiyohara et al., 1975; Mackie, 1973; Mackie and Adron, 1978). Sin embargo, las investigaciones pioneras de Burrells et al. (2001a, 2001b) dieron como resultado una mayor atención sobre la suplementación con nucleótidos para peces, ya que sus estudios indicaron que esta suplementación mejoró la resistencia de los salmónidos a las infecciones virales, bacterianas y parasitarias, así como también mejoró la eficacia de la vacunación y la capacidad de osmorregulación. Hasta la fecha, las investigaciones relacionadas con la nutrición de nucleótidos en los peces han mostrado resultados beneficiosos consistentes y alentadores en el crecimiento y el manejo de la salud de los peces (Bowyer et al., 2019; El-Nokrashy et al., 2021; Guo et al., 2017;

Hossain et al., 2016b; Reda et al., 2018; Shiau et al., 2015; Tahmasebi-Kohyani et al., 2011; Xu et al., 2015; Yin et al., 2015).

Otro grupo de aditivos alimentarios son las proteínas funcionales que se utilizan ampliamente en muchas especies, incluidos cerdos, aves de corral, rumiantes, animales de compañía y en la acuicultura. Una parte de estas proteínas funcionales proviene de los subproductos del procesamiento industrial de ganado, aves y pescado, los cuales tienen un potencial de conversión en productos útiles de mayor valor, como los hidrolizados de proteínas, con aplicaciones interesantes en la alimentación animal. Las bajas cantidades de hidrolizados de proteínas animales incluidas en los alimentos acuícolas pueden mejorar la tasa de crecimiento y la conversión del alimento de peces y crustáceos (Martínez-Alvarez et al., 2015), ya que proporcionan péptidos altamente digeribles y péptidos bioactivos, así como AA específicos (p. ej., ácido glutámico) para conferir funciones nutricionales y fisiológicas o reguladoras en los animales (Hou et al., 2017). Los hidrolizados de proteínas animales también se pueden incorporar en las dietas para mejorar la inmunidad no específica de los peces (Martínez-Alvarez et al., 2015). La mucosa porcina hidrolizada (MPH) es un nuevo tipo de fuente de proteína animal nutritiva funcional. La MPH se obtiene durante el proceso de producción de heparina (fármaco anticoagulante) a partir de tejidos digestivos endoteliales y mucosos limpios de cerdos, la cual se digiere enzimáticamente y se seca, y luego se rocía en un portador de harina de soja para facilitar el manejo (Mateos et al., 2014). Estudios recientes han demostrado el uso de MPH para el beneficio del desarrollo de varias especies de peces, como la carpa común, *Cyprinus carpio* (Gao et al., 2020); tilapia nilótica, *Oreochromis niloticus* (dos Santos Cardoso et al., 2021); mero híbrido (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) (Yang et al., 2021a) y lubina negra, *Micropterus salmoides* (Yu et al., 2023).

Existe un interés creciente en el uso de las microalgas como aditivos alimentarios funcionales, debido a que estos microorganismos se caracterizan por un contenido relativamente alto, tanto de proteínas como de ácidos grasos poliinsaturados (Huerlimann et al., 2010; Renaud et al., 1994). Esta alternativa está respaldada por numerosos estudios que demuestran que el uso de microalgas no solo no produce efectos perjudiciales en los peces en términos de bienestar, sino que también podría ser beneficioso en varios aspectos, como la eficiencia alimenticia y el bienestar animal (Perera et al., 2020), la salud

intestinal y/o la protección de la mucosa intestinal, mejorando funciones como la absorción y asimilación de nutrientes (Jorge et al., 2019; Vizcaíno et al., 2018) o la pigmentación (Galafat et al., 2020; Pulcini et al., 2021). Otro aspecto de la inclusión de microalgas en los alimentos para peces estudiado recientemente es su efecto sobre la calidad física del alimento, ya que se encontró que éstas aumentan su dureza y durabilidad y reducen la pérdida de grasa del gránulo (Alcaraz et al., 2021). Todas estas particularidades han llevado a una mayor exploración del uso de las microalgas con el fin de proporcionar un beneficio adicional para la salud de los peces. En investigaciones llevadas a cabo en doradas y truchas, la inclusión de microalgas en las dietas indica que mejora el crecimiento, la condición fisiológica y la eficiencia alimenticia (Liu et al., 2022; Molina-Roque et al., 2022; Perera et al., 2020), así como también favorece la inmunidad y la capacidad antioxidante (Cerezuela et al., 2012b; Chen et al., 2022; Galafat et al., 2020; Reyes-Becerril et al., 2013), la modulación de la expresión génica intestinal (Cerezuela et al., 2013) y la funcionalidad y ultraestructura intestinal (Galafat et al., 2020).

En resumen, una buena parte de las investigaciones ha proporcionado evidencia sobre los efectos beneficiosos de los aditivos alimentarios en el crecimiento, la capacidad de utilización del alimento, las respuestas inmunitarias y la calidad del producto final en la acuicultura. Los aditivos alimentarios son una parte integral de las formulaciones modernas de piensos en la acuicultura y el mercado mundial de estos aditivos alimentarios ha crecido significativamente, por lo que los investigadores exploran continuamente los aditivos alimentarios, los introducen en el mercado y los incorporan a los piensos comerciales. En la Tabla 2 se presenta un compendio de las últimas investigaciones relacionadas con la suplementación de piensos con aditivos alimentarios. Los valores resaltados en negrita corresponden a los niveles de inclusión de aditivos que mostraron mejores resultados.

Tabla 2. Investigaciones recientes sobre la suplementación dietética con aditivos alimentarios en peces.

Autores	Aditivos	Dosis y/o régimen de alimentación	Duración	Especies	Tamaño inicial	Efectos
			Nucleótidos			
Tahmasebi-Kohyani et al. (2011)	Nucleótido (Optimun, Chemoforma, Augst, Switzerland)	0,5; 1; **1,5** y **2** g kg^{-1} dieta	8 semanas	Trucha arco iris, *Oncorhynchus mykiss*	23 g	Crecimiento↑, respuesta inmune humoral↑
Shiau et al. (2015)	Nucleótido (Rovimax NX, DSM nutritional Products, Basel, Switzerland)	**120**, **240**, 360, 480 y 600 mg kg^{-1} dieta	10 semanas	Tilapia híbrida, *Oreochromis niloticus* × *Oreochromis aureus*	0,15 g	Respuesta inmune↑, supervivencia frente a *Streptococcus iniae*↑
Xu et al. (2015)	Mezcla de nucleótidos (Biotogether, Nanjing, China)	0,15; 0,30; **0,60**; y 1,20 g 100 g^{-1} dieta	8 semanas	Tilapia híbrida, *Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂	8,02 g	Crecimiento↑, morfología intestinal↑, estado antioxidante↑, respuesta inmunitaria no específica↑
Yin et al. (2015)	Nucleótidos de levadura (Nanjing Master Biotechnology Co. Ltd., Nanjing, China)	150, 300, **450**, **600**, 750 y 900 mg kg^{-1} dieta	50 días	*Ancherythroculter nigrocauda*	23,30 g	Crecimiento↑, contenido intramuscular de proteínas y grasas↑, inmunidad↑
Hossain et al. (2016)	Mezcla de nucleótidos purificados (Sigma Aldrich Co., St. Louis, MO, USA)	0,5; **1,0**; **1,5**; 2,0 y 2,5 g kg^{-1} dieta	56 días	Dorada del Japón, *Pagrus major*	2,6 g	Crecimiento↑, inmunidad humoral↑, resistencia al estrés↑

Hossain et al. (2016b)	Subproductos de nucleótidos (Ajinomoto Co., Inc., Tokio, Japan)	**1** y 3 g 100 g^{-1} dieta	60 días	Dorada del Japón, *Pagrus major*	2,28 g	Crecimiento↑, respuesta inmunitaria no específica↑
Guo et al. (2017)	Mezcla de nucleótidos (Sigma Chemical)	0,05; **0,10**; 0,15; y 0,20 g 100 g^{-1} dieta	4 semanas	Pez cebra, *Danio rerio*	100 mg	Crecimiento↑, morfología intestinal↑, actividad lipasa↑
Reda et al. (2018)	Nucleótido (Nucleoforce Fish™, Bioiberica, Spain)	0,05; 0,15 y **0,25** g 100 g^{-1} dieta	30 días	Tilapia nilótica, *Oreochromis niloticus*	42,90 g	Hematología↑, inmunidad no específica↑, actividad antioxidante↑, expresión génica↑, resistencia a *Aeromonas sobria*↑
Bowyer et al. (2019)	Nucleótido (Laltide®, Lallemand Animal Nutrition, UK)	0,15 y **0,30** g 100 g^{-1} dieta	6 semanas	Lubina, *Dicentrarchus labrax*	62,19 g	Crecimiento↑, morfología intestinal↑
El-Nokrashy et al. (2021)	Nucleótido (Nucleoforce Fish™, Bioiberica, Spain)	**250** y 500 mg kg^{-1} dieta	150 días	Dorada, *Sparus aurata*	0,35 g	Crecimiento↑, riqueza microbioma intestinal↑, enzimas metabólicas↑, expresión génica específica↑
		Mucosa Porcina Hidrolizada				
dos Santos Cardoso et al. (2021)	Proteína de mucosa porcina hidrolizada	**200** g kg^{-1} dieta	30 días	Tilapia nilótica, *Oreochromis niloticus*	69,08 g	Crecimiento↑

Yang et al. (2021)	Mucosa porcina hidrolizada	**30**, 60, 90 y 120 g kg^{-1} dieta	56 días	Mero híbrido, *Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂	7,50 g	Crecimiento↑, actividad enzimas digestivas↑, transporte de AA y péptidos pequeños↑
Yu et al. (2023)	Proteína de mucosa intestinal hidrolizada enzimáticamente (Wuxi Tongwei Feedstuffs Co., Ltd., Wuxi, China)	**3,50**; **7,30**; 11,00 y 14,70 g kg^{-1} dieta	95 días	Lubina negra, *Micropterus salmoides*	24 g	Crecimiento↑
			Microalgas			
Cerezuela et al. (2012a)	*T. chuii* y *P. tricornutum*	**100** g kg^{-1} dieta	4 semanas	Dorada, *Sparus aurata*	50 g	Efecto inmunoestimulante↑
Cerezuela et al. (2012b)	*N. gaditana, T. chuii* y *P. tricornutum*	**50** y **100** g kg^{-1} dieta	2 y 4 semanas	Dorada, *Sparus aurata*	100 g	Estimulación actividades de defensa↑
Reyes-Becerril et al. (2013)	**Ensilaje de *Navicula* sp. + *Lactobacillus sakei* 5-4** y *Navicula* sp. liofilizada	**106 UFC g^{-1}** y 100 g kg^{-1}, respectivamente	4 semanas	Dorada, *Sparus aurata*	80 g	Estado inmunológico↑, expresión de genes inmunológicos↑
Vizcaíno et al. (2018)	*T. lutea, N. gaditana* o *S. almeriensis* (Estación Experimental "Las Palmerillas", Fundación Cajamar, Almería, Spain)	**150** g kg^{-1} dieta	85 días	Lenguado senegalés, *Solea senegalensis*	11,4 g	Crecimiento↑, capacidad de absorción de la mucosa intestinal↑

Jorge et al. (2019)	Microalga liofilizada *N. gaditana* (Buggypower, Lda., Portugal)	**5**; **7,5** y **15** g kg^{-1} dieta	137 días	Dorada, *Sparus aurata*	56,6 g	Riqueza de la microbiota↑
Perera et al. (2020)	Productos a base de microalgas (LifeBioencapsulation S.L., Almería, Spain)	**5** y **10** g kg^{-1} dieta	187 días	Dorada, *Sparus aurata*	12–13 g	Crecimiento↑, eficiencia alimenticia↑
Galafat et al. (2020)	*Arthrospira* sp. hidrolizada enzimáticamente	**20** y **40** g kg^{-1} dieta	128 días	Dorada, *Sparus aurata*	20 g	Ultraestructura y funcionalidad intestinal↑, pigmentación muscular↑, capacidad antioxidante↑
Liu et al. (2022)	*Chlorella sorokiniana* (Center for Microalgal Biotechnology and Biofuels, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China)	8,8; 18; 27 y **36** g 100 g^{-1} dieta	8 semanas	Trucha arco iris, *Oncorhynchus mykiss*	67,65 g	Crecimiento↑, metabolismo↑, salud intestinal↑
Molina-Roque et al. (2022)	Productos a base de microalgas (LifeBioencapsulation S.L., Almería, Spain)	**10** g 100 g^{-1} dieta	41 días	Dorada, *Sparus aurata*	~ 182 g	Crecimiento↑, condición fisiológica↑

Chen et al. (2022)	*Chlorella sorokiniana* (Hydrobiology, Chinese Academy of Science, Wuhan, China)	**5** y **10** g 100 g^{-1} dieta	90 días	Trucha arco iris, *Oncorhynchus mykiss*	165,3 g	Crecimiento↑, capacidad antioxidante↑, respuesta inmune↑, supervivencia a *Aeromonas salmonicida*↑

El uso de ingredientes alternativos a la harina de pescado se hace cada vez más imprescindible para alcanzar una acuicultura económica y ambientalmente sostenible, y para conocer los efectos causados por estos ingredientes se partirá de la hipótesis: **Es beneficioso para el crecimiento de peces carnívoros, el uso de fuentes proteicas vegetales, animales y aditivos en piensos con alta sustitución de harina de pescado.**

La verificación-refutación de esta hipótesis se realiza a partir del estudio de altas sustituciones de HP en piensos para peces carnívoros. Como punto de partida, el primer capítulo estudia la máxima sustitución de HP en piensos para trucha arco iris para determinar el máximo nivel de sustitución sin que afecte el crecimiento y la salud de los peces. El segundo y tercer capítulo analizan los efectos de la inclusión de aditivos y el uso de fuentes proteicas vegetales y animales en piensos para doradas sobre el crecimiento, la eficiencia de utilización del alimento y la histología intestinal.

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JUSTIFICACIÓN Y OBJETIVOS

La acuicultura es una de las industrias de producción animal con mayor crecimiento a nivel mundial y la idoneidad de ésta depende de la reducción del uso de la HP. A pesar de que ya se han evaluado con éxito varias alternativas de origen animal y vegetal para reducir la gran dependencia de la HP, se sabe que las fuentes proteicas vegetales contienen cantidades relativamente altas de FAN y baja digestibilidad de nutrientes (Francis et al., 2001; Gatlin III et al., 2007b; Kaushik and Hemre, 2008), que no están asociados con los hábitos alimenticios naturales de las especies marinas carnívoras (Kaushik and Hemre, 2008). Sin embargo, el potencial de las harinas de proteína vegetal como sustituto parcial de HP se ha establecido en la acuicultura marina de carnívoros (Alami-Durante et al., 2010; Burr et al., 2012; Martínez-Llorens et al., 2012a; Sánchez-Lozano et al., 2009; Santigosa et al., 2008; Tomás-Almenar et al., 2020). Del mismo modo, las proteínas animales también representan un gran potencial como posibles ingredientes alternativos para la sustitución de HP (Cardinaletti et al., 2022; Fernández-Alacid et al., 2022; Fontinha et al., 2021; Psofakis et al., 2020; Randazzo et al., 2021a).

Debido a estos cambios en la alimentación natural de especies carnívoras y para asegurar que los nutrientes de la dieta sean ingeridos, digeridos, absorbidos y transportados a las células con eficiencia, se está utilizando una diversidad cada vez mayor de aditivos alimentarios (Encarnação, 2016). Tal es el caso del uso de los nucleótidos en trucha (Tahmasebi-Kohyani et al., 2011) y en doradas (El-Nokrashy et al., 2021); la mucosa porcina hidrolizada (MPH) en peces omnívoros (dos Santos Cardoso et al., 2021; Gao et al., 2020) y carnívoros (Yang et al., 2022; Yu et al., 2023); y las microalgas en truchas (Chen et al., 2022; Liu et al., 2022) y en doradas (Galafat et al., 2020; Jorge et al., 2019; Molina-Roque et al., 2022; Perera et al., 2020).

En virtud de ello, el objetivo principal de este trabajo fue estudiar el efecto de la alta sustitución de HP en piensos para peces carnívoros, inclusive la sustitución total de ésta, así como la inclusión de aditivos alimentarios sobre el crecimiento, el aprovechamiento nutritivo y la salud de truchas y doradas, dos especies representativas en la producción acuícola mundial.

Para llevar a cabo este objetivo, se plantearon los siguientes objetivos específicos:

- Estudiar el efecto de la sustitución total de HP en piensos para trucha arco iris sobre el crecimiento, supervivencia y parámetros nutritivos.

- Establecer el impacto sobre la salud intestinal de esta sustitución total en la misma especie, en términos de parámetros histológicos del intestino, expresión de genes relacionados con procesos inflamatorios y respuestas inmunes, e integridad epitelial en el intestino.
- Evaluar los efectos de la inclusión de dos aditivos alimentarios en diferentes dosis, un concentrado de nucleótidos y mucosa porcina hidrolizada, en un pienso para doradas basado 100% en proteína vegetal sobre el crecimiento, la eficiencia alimenticia, la digestión proteica e histología intestinal.
- Evaluar el efecto de la sustitución de HP por una mezcla de proteínas vegetales y animales (harina de cerdo ibérico), así como la inclusión de la microalga *Isochrysis* aff. *galbana* (T-Iso) en piensos para doradas sobre el crecimiento, la eficiencia de utilización del alimento, los índices biométricos y las eficiencias de retención de energía, proteína y aminoácidos.

Los análisis realizados en cada uno de los experimentos se resumen en la Figura 1.

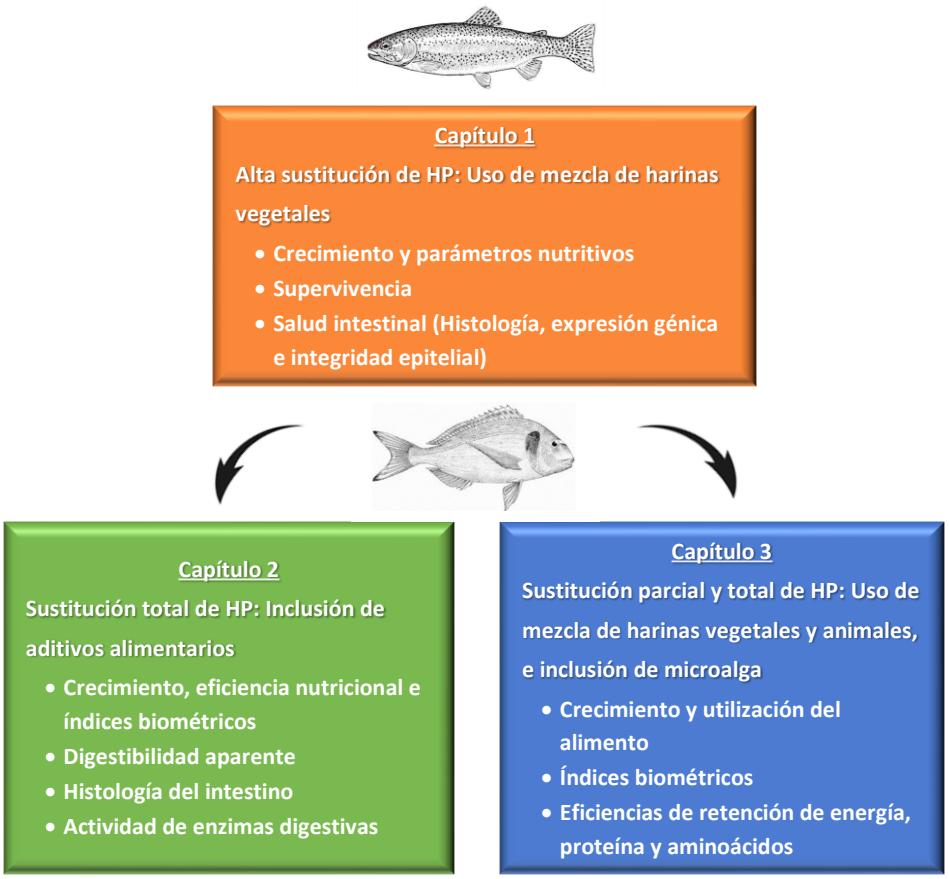


Figura 1. Resumen de los experimentos llevados a cabo en la presente tesis doctoral (elaboración propia).

Conjuntamente, se realizó un estudio económico y un análisis ambiental de la sustitución de harina de pescado, con el fin de evaluar la rentabilidad de los piensos y la sostenibilidad de la alimentación de los peces, respectivamente.

CAPÍTULO 1

Successful inclusion of high vegetable protein sources in feed for rainbow trout without decrement in intestinal health

Successful inclusion of high vegetable protein sources in feed for rainbow trout without decrement in intestinal health

Glenda Vélez-Calabria, David Sánchez Peñaranda, Miguel Jover-Cerdá, Silvia Martínez Llorens and Ana Tomás-Vidal

Aquaculture and Biodiversity Research Group. Institute of Science and Animal Technology. Universitat Politècnica de València, Valencia, Spain.

Animals 2021, *11*(12), 3577

Abstract

The aquaculture of carnivorous fish is in continuous expansion, which leads to the need to reduce the dependence on fishmeal (FM). Plant proteins (PP) represent a suitable protein alternative to FM and are increasingly used in fish feed. However, PP may lead to stunted growth and enteritis. In the current study, the effect of high FM substitution by PP sources on the growth, mortality and intestinal health of rainbow trout (*Oncorhynchus mykiss*) was evaluated in terms of the histological intestine parameters and expression of genes related to inflammation (*IL-1β, IL-8* and *TGF-β*) and immune responses (*Transferrin, IgT* and *IFN-γ*). The results show that a total substitution registered lower growth and survival rates, probably due to a disruption to the animal's health. Confirming this hypothesis, fish fed FM0 showed histological changes in the intestine and gene changes related to inflammatory responses, which in the long-term could have triggered an immunosuppression. The FM10 diet presented not only a similar expression to FM20 (control diet), but also similar growth and survival. Therefore, 90% of FM substitution was demonstrated as being feasible in this species using a PP blend of wheat gluten (WG) and soybean meal (SBM) as a protein source.

Keywords: intestinal tract, fishmeal substitution, amino acid supplement, interleukins, immune system, inflammation response

1. Introduction

In order to improve sustainability and profitability, the aquaculture industry has followed the tendency to replace fishmeal (FM), or at least reduce it, without a decrement of nutritional quality and intestinal health [1]. As a consequence, much of the research done on carnivorous species, including rainbow trout, have been tested to evaluate the effect of FM substitution by alternative protein sources, such as soybean meal (SBM) or soy protein concentrates [2,3], rice protein concentrate or canola protein concentrate [4,5]. Alternative proteins of animal origin have also been tested, such as feather meal and meat and bone meal [6], krill [7] or bacterial protein [8], but the best growth results have been obtained with mixtures of vegetable protein ingredients [2,9,10].

Soybean protein products are considered one of the most workable alternatives due to their reasonable price and a steady supply of soybeans [11], although previous studies on high contents of soybean in diets reported alterations in the immune system of the intestinal track [12,13]. Until now, the use of SBM has been limited in feeds for salmonids, likely due to its relatively low protein content; however, concentrates produced from soybean (soybean protein concentrates; SPCs) have solved this problem with a mean crude protein (CP) content of 65–70% [14]. In fact, by including SPC as an alternative protein source it has been possible to achieve the maximum FM replacement (33 to 100% replacement) without affecting growth performance or nutrient utilization in rainbow trout [15].

Although the highest replacement has been achieved using plant proteins (PP), this protein resource presents some limitations in carnivorous species, such as the high carbohydrate content, deficiency in certain essential amino acids (EAA; e.g., methionine (Met), lysine (Lys), tryptophan (Trp), threonine (Thr) and arginine (Arg)) or low palatability. Another possible negative factor is the presence of antinutritional factors (ANFs), such as protease inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamins and allergens [16,17]. Therefore, the inclusion of high levels of plant ingredients may affect the health conditions of fish. For example, a decrease in innate immune response and an inflammatory response upon feeding high amounts of PP ingredients has been reported in rainbow trout [18] and in other carnivorous species such as gilthead seabream, *Sparus aurata* [19,20], Senegalese sole (*Solea senegalensis*) [21] or hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) [22].

Previous studies with a total FM substitution with raw materials of vegetable origin have also been performed in carnivorous species, obtaining, during short feeding periods (64 days), similar growth rates to FM feeds [23]. However, in the long-term (>12 weeks), the growth rate decreased accompanied by high mortality associated with intestinal and hepatic histomorphological alterations [24], disorders of the immune state [20] or microbial imbalances [25]. One of main factors of this negative effect on health could be the presence of ANFs in PP. ANFs can affect the digestibility and absorption of nutrients [26], as well as intestinal integrity [27], changing the microbial abundance and species richness [25]. In contrast, the negative effects of FM substitution on health status can be partially corrected by the inclusion of EAA supplements in the diets. AA supplementation is required in feed with high FM substitution in order to restore the appropriate AA profile to the target species [28]. For example, Arg supplementation showed a significant effect on non-specific immune responses in Nile tilapia *Oreochromis niloticus* juveniles [29] and golden pompano *Trachinotus ovatus* juveniles [30]. Cheng et al. [31] evaluated the effects of a diet with Arg and glutamine (Gln) supplementation in diets for juvenile red drum (*Sciaenops ocellatus*), confirming the enhancer action of AA supplementation on the immune system as well as on intestine structure with an increase in microvillus height. In European seabass juveniles (*Dicentrarchus labrax*) [32], AA supplementation enhanced the antioxidant defense response. Finally, Feng et al. [33] reported that methionine hydroxy analogue (MHA) supplementation promoted the antioxidant defense in the intestine and hepatopancreas of juveniles Jian carp (*Cyprinus carpio* var. Jian).

In rainbow trout, high levels of PP sources or alternative protein blends (animal and vegetable) have not compromised the growth performance, even with FM substitutions higher than 75% [9,34–38]. Nevertheless, high FM substitutions may negatively affect fish intestinal health, as has been reported by Santigosa et al. [9] with an increase in relative intestinal length (RIL) in diets with FM substitution above 75%. Previous studies replacing FM with SBM in rainbow trout showed changes and damages to the digestive tract [3,39,40]. Romarheim et al. [41] found that rainbow trout fed a diet with 30% SBM showed a development of enteritis with a general progression of reduced mucosal fold height and increased lamina propria. Jalili et al. [42] assessed higher inclusions of other vegetable protein sources (70 and 100%; wheat gluten (WG), corn gluten (CC) and SBM), finding lower growth and depression of immune response. In other species, such as

Atlantic salmon (*Salmo salar*), substitution of FM with SBM provided similar results [43].

In conclusion, the effect of high levels of plant ingredients on fish health conditions still remains in the process of study, and an integrative approach is needed to clarify the interactions between nutrition and the immune system in order to understand the physiological processes involved. Thus, the objective of the current study was to achieve the maximum FM substitution using as an alternative protein the mixture of PP (wheat meal (WM), WG and SBM) in rainbow trout (*Oncorhynchus mykiss*) without affecting growth, survival and intestinal health. With this aim, the intestinal health status of rainbow trout will be analyzed in terms of intestinal histology and the expression of genes related to inflammatory processes, immune systems and epithelial integrity in the anterior and posterior intestine.

2. Materials and methods

The experimental protocol was reviewed and approved by the Ethics and Animal Welfare Committee of the Polytechnic University of Valencia (Official bulletin No. 80 of 06/2014), following Royal Decree 53/2013 and the European Directive 2010/63/EU on the protection of animals used for scientific research, with the purpose of minimizing the suffering of animals.

2.1. Production system

The growth trial was performed in a recirculation freshwater system (65 m^3 capacity), with a rotary mechanical filter and a gravity biofilter (approximately 6 m^3), equipped with nine cylindrical fiberglass tanks of 1750 L. All tanks were equipped with aeration. Water temperature was 15.5 ± 0.1 °C, dissolved oxygen was 9.3 ± 0.3 mg L^{-1} and pH ranged from 8.4 to 8.6. All these parameters were measured on a daily basis from Monday to Saturday. Photoperiod was natural, and all tanks had similar light conditions. The experimental period was 77 days (from November 2016 to February 2017).

2.2. Fish and Experimental Design

Rainbow trout from a fish farm (Zarzalejo, Albacete, Spain) were transported alive to the Aquaculture Laboratory of Polytechnic University of Valencia and randomly distributed in experimental tanks. Prior to the feeding trial, all fish were acclimatized for two weeks and fed a standard diet provided by the fish farm. After these two weeks, the fish were

weighed (13.4 ± 0.3 g) and distributed into groups of 60 animals per tank. All fish were weighed every 28 days approximately. Previously, fish were anaesthetized with 30 mg L^{-1} of clove oil (Guinama®, Valencia, Spain) containing 87% eugenol. The fish were not fed for one day before weighing.

In order to evaluate gene expression throughout the intestine tract at 11 weeks after the start of the experiment, three fish digestive tract samples per tank were taken. Euthanasia was performed by anesthetic overdose (benzocaine 60 ppm for 10 min), and then fish were dissected to obtain the digestive tract. Two different sections were collected, based on the separation in sections proposed by Venou et al. [44]: anterior tract (the upper intestine, section between the stomach's pyloric sphincter and the first bend of the digestive tract) and posterior tract of the intestine (section after this narrowing and the anus, obtaining two replications (portions of 16 mm^2) of each section per fish. The fragments of the sections were stored in RNA later® (Qiagen, Valencia, Spain) for 24 h at 4 °C and subsequently at −20 °C until gene expression analysis.

2.3. Diets and feeding

The formulation and proximate composition of experimental diets are shown in Table 1. The PP blend (WG and SBM) was included in experimental diets at three dietary levels such that 80% (FM20), 90% (FM10) and 100% (FM0) of the FM was replaced. FM20 was considered the control diet. The three experimental diets were formulated to contain 44% crude protein (CP) and 19% crude lipid (CL), similar to commercial trout diets that contain CP levels ranging from 42% to 48% depending on fish size [45] and 19% CL [36]. Each diet was assayed in triplicate tanks and randomly assigned to 9 tanks. Diets were supplemented with feed-grade Lys, Met, valine (Val) and Tau based on the nutritional requirements for trout recommended by NRC [46]. The profile of dietary amino acids is shown in Supplementary Table S1.

Table 1. Formulation and proximate composition of experimental diets.

	Experimental Diets		
	FM0	**FM10**	**FM20**
Ingredients (g kg^{-1})			
Fishmeal [1]	0	100	200
Wheat meal [2]	116	158	201
Wheat gluten [3]	214	201	180
Soybean meal [4]	400	300	200
Soybean oil	88	89	90
Fish oil	100	91	82
Calcium phosphate	38	33	28
Taurine [5]	20	10	5
Valine [5]	2	0	0
L- Methionine [5]	4	2	0
L-Lysine Clh [5]	8	6	4
Vitamin-mineral mix [6]	10	10	10
Proximate composition (g kg^{-1} on dry matter)			
Dry matter (DM)	913	912	912
Crude protein (CP)	446	440	434
Crude lipid (CL)	191	190	192
Ash	42	68	75
Calculated values			
Carbohydrates (CHO) [7]	322	302	299
Calculated GE (MJ kg^{-1}) [8]	22.5	22.6	23.3

[1] Fishmeal: (93.2% DM, 70.7% CP, 8.9% CL, 15.1% Ash); Vicens I Batllori S.L., Girona, Spain. [2] Wheat meal (92.4% DM, 17.1% CP, 2.4% CL, 78.3% CHO, 2.4% Ash); Piensos Y Cereales Desco, Museros, Valencia, Spain. [3] Wheat gluten: (70.9% CP, 1.3% CL, 34.1% CHO, 1.5% Ash); Dadelos Agrícola, Valencia, Spain. [4] Soybean meal: (88.2% DM, 49.9% CP, 2.2% CL, 7.1% Ash); Piensos Y Cereales Desco, Valencia, Spain. [5] Taurine, Valine, L-Methionine and L-Lysine: Guinama S.L.U. [6] Vitamin and mineral mix (values are g kg^{-1} except those in parentheses): Premix: 25; Choline, 10; DL-α-tocopherol, 5; ascorbic acid, 5; $(PO_4)_2Ca_3$, 5. Premix composition: retinol acetate, 1 000 000 IU kg^{-1}; calcipherol, 500 IU kg^{-1}; DL-α-tocopherol, 10; menadione sodium bisulphite, 0.8; thiamine hydrochloride, 2.3; riboflavin, 2.3; pyridoxine hydrochloride, 15; cyanocobalamine, 25; nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07; ascorbic acid, 75; inositol,15; betaine, 100; polypeptides 12. Zn, 5; Se, 0.02; I, 0,5; Fe, 0.2; CuO, 15; Mg, 5.75; Co, 0.02; Met, 1.2; Cys, 0.8; Lys, 1.3; Arg, 0.6; Phe, 0.4; Trcp, 0.7; excpt. 1000 g. [7] Carbohydrates (g kg^{-1}) = 100−CP (g kg^{-1})−CL (g kg^{-1})−Ash (g kg^{-1}). [8] Calculated energy (MJ kg^{-1}) = [(51.8 × C)–(19.4 × N)]. The C–N was analyzed by way of the Dumas principle (TruSpec CN; Leco Corporation, St. Joseph, MI, USA). Calculated according to Brouwer [47].

Diets were prepared by cooking extrusion processing with a semi-industrial twin-screw extruder (CLEXTRAL BC-45, Firminy, St Etienne, France). The processing conditions were as follows: 100 rpm speed screw, temperature of 110 °C, 40–50 atm pressure and pellets with diameters from 2 to 3 mm. Experimental diets were assayed in triplicate. Fish were fed by hand twice a day (9:00 and 17:00 h) from Monday to Saturday until apparent

satiation. Pellets were distributed slowly, allowing all fish to eat, and the total amount of feed distributed was recorded.

2.4. Analysis of intestinal health status

In order to evaluate the effect of high levels of FM substitution on rainbow trout intestinal health, intestinal histology, the gene expression of inflammatory and immune processes and epithelial integrity in the anterior and posterior intestine was analyzed.

2.4.1. Intestinal histology

At the end of the experiment, intestine samples from five fish from each tank were collected and dissected into small pieces and preserved in phosphate-buffered formalin (4%, pH 7.4). All of the formalin-fixed tissues were routinely dehydrated in ethanol, equilibrated in ultraclean and embedded in paraffin according to standard histological techniques. Transverse sections were cut with a Microtome Shandon Hypercut to a thickness of 5 μm and stained with Alcian blue for gut examination. A total of eighteen sections per treatment were examined under a light microscope (Nikon, Phase Contrast Dry JAPAN 0.90).

For the measurements and observations of the intestine, a combination of criteria reported by several authors was followed [9,20], and the following parameters were measured: serous layer (SL), muscular layer (ML), submucous layer (SML), villi length (VL), villi thickness (VT), intra villi space and lamina propria length (LP) and thickness. In addition, a quantification of goblet cells (GC) was performed by counting the number of GC present in each villus. A total of six villi per section were used.

2.4.2. Gene expression

RNA extraction

Total ribonucleic acid (RNA) was extracted from the anterior and posterior section of the intestine by traditional phenol/chloroform extraction, using the Trizol reagent (Invitrogen, Barcelona, Spain), and then purified and treated with DNase I using the NucleoSpin® RNA Clean-up XS kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. The concentration, quality and integrity of the total RNA were evaluated with a NanoDrop 2000C spectrophotometer (Fisher Scientific SL, Madrid, Spain). Only samples that obtained an absorbance ratio A260/280 between 1.8–2.0 and A260/230 greater than 2.0 were included in the analysis. The RNA samples were

stored at –80 °C until the stage of complementary deoxyribonucleic acid synthesis (cDNA) to avoid RNA degradation.

Subsequently, the cDNA was synthesized from 1 µg of RNA using the qScript Flex cDNA kit (Quanta BioScience, Beverly, MA, USA), according to the manufacturer's instructions and using the Applied Biosystems 2720 thermal cycler. The thermocycler conditions were 22 °C during 5 min, 42 °C for 30 min and 85 °C for 5 min. Once the cDNA was obtained, it was stored at –20 °C until the gene expression was analyzed.

2.4.3. Quantitative real-time RT-PCR (qPCR)

The real-time polymerase chain reaction (RT-qPCR) consists of the amplification of cDNA prepared using the reverse transcription (RT) of messenger ribonucleic acid (mRNA) by way of quantitative real-time polymerase chain reaction (qPCR), which is a tool commonly used to study and evaluate gene expression [48].

Reference and target genes

In order to select the best reference gene for the experiment, two candidate reference genes were selected: *Elongation Factor 1α* (*ELF-1α*) and *β-actin*, whose primer sequences are shown in Table 2. The stability was analyzed thanks to the BestKeeper program [49] based on the Ct values obtained. The expression of three inflammatory genes related to primary inflammation, *Interleukin 1β* (*IL-1β*)*, Interleukin 8* (*IL-8*) and *Transforming Growth Factor beta* (*TGF-β),* was analyzed. These inflammation-stimulating molecules are immuno-regulatory genes commonly used in rainbow trout. *IL-1β* was the first cytokine cloned in fish and has been identified in several teleost species, including salmonids [50]. It has a wide range of target cells and plays a fundamental and central role both in the initiation and in the regulation of inflammation [51]. *IL-8* is another pro-inflammatory cytokine that is involved in chemotaxis and in the activation of the different cell types involved in inflammation. It is known especially for being able to promote the adhesion of the monocytes and neutrophils that are in the blood circulation to the endothelial cells that form the blood vessels, helping them to pass from the blood to the inflamed tissue, so that they can exercise their action [52]. *TGF-β* is also another cytokine involved in immune and inflammatory processes, since it regulates the proliferation of the cells of the defense system (T and B lymphocytes), as well as the expression of some immunoglobulins. In addition, it regulates the expression of adhesion

molecules. This molecule also acts as a chemoattractant for fibroblasts, monocytes and neutrophils [53]. It is believed that these three types of cytokines contribute to the hosting of defense mechanisms in response to colonization or bacterial invasion [54].

We also studied three immune genes related to the later response of the immune system: *Transferrin, Immunoglobulin T (IgT)* and *Interferon gamma (IFN-γ). Transferrin* is a lectin that binds to iron and has antimicrobial properties and, therefore, plays an important role in the pathology of many bacterial infections, limiting the amount of this essential endogenous nutrient available to invading pathogens and therefore, its ability to reproduce [55]. *IgT*, on the other hand, is an immunoglobulin specialized in immune responses of the intestinal mucosa [56]. Finally, *IFN-γ* is considered an important pro-inflammatory cytokine that plays an important regulatory role in both the innate and adaptive immune response in teleost cells [57], since it plays an important role in the activation of macrophages by increasing their phagocytic capacity. The primers used for the amplification reaction are shown in Table 2.

Table 2. General information about the target and reference genes.

Category	Gen/Protein Description	Abbrev.	Primer Sequence		GenBank	BP Size	Reference
			Forward	Reverse			
Housekeeping genes	*Beta-actin*	*β-actin*	GCCGGCCGCGACCTCACAGACTAC	CGGCCGTGGTGGTGAAGCTGTAAC	EZ908974	73	Evenhuis and Cleveland, [58]
	Elongation factor 1-alpha	*ELF-1α*	ACCCTCCTCTTGGTCGTTTC	TGATGACACCAACAGCAACA	AF498320	63	Kania et al. [59]
	Transferrin		5'CCACCTCCAGGGCCATTAAATG3'	5'ATCCACCGCTATGGCATCTGCC3'	D89083		Talbot et al. [60]
Immune	*Immunoglobulin T*	*IgT*	AACATCACCTGGCACATCAA	TTCAGGTTGCCCTTTGATTC	AY870265	80	Evenhuis and Cleveland, [58]
	Interferon gamma	*IFN-γ*	CTGTTCAACGGAAACCCTGT	AACACCCTCCGATCACTGTC	NM 001160503	62	Evenhuis and Cleveland, [58]
	Interleukin-1 beta	*IL-1β*	ACATTGCCAACCTCATCATCG	TTGAGCAGGTCCTTGTCCTTG	AJ223954	91	Pérez-Sánchez et al. [61]
Inflammatory	*Interleukin 8*	*IL-8*	CTCGCAACTGGACTGACAAA	TGGCTGACATTCTGATGCTC	AJ279069	148	Evenhuis and

					Cleveland, [58]
Transforming growth factorTGF-β beta	TCCGCTTCAAAA TATCAGGG	TGATGGCATTTTCA TGGCTA	X99303	71	Evenhuis and Cleveland, [58]

RT-qPCR conditions and gene expression quantification

All qPCR assays and expression analyses were performed using Real-Time PCR Applied Biosystems 7500 with SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) as a detection system. After initial activation of the Taq polymerase at 95 °C for 10 min, 42 cycles of PCR were performed using the Light Cycler with the following cycle conditions: 95 °C for 10 s and 60 °C for 30 s. To evaluate the specificity of the assay, a melting curve analysis was performed directly after the PCR by slowly increasing the temperature (1 °C min^{-1}) from 60 to 95 °C, with a continuous record of the changes in the intensity of the fluorescent emission.

These assays were carried out in 96-well plates, and each reaction was carried out in duplicate. The volume per well was 20 µL, of which 5 µL were cDNA (diluted 1:40), 10 µL SYBR Green PCR Master Mix, 1 µL per primer (forward and reverse, 5 mM), 1 µL of water and 2 µL of reference fluorophore ROX™ (6-carboxy-X-rhodamine, 100 nM). In addition, a calibrator and a blank were included in all the plates. The interplate coefficient of variation was 1.94%. The reference genes and those of interest in all the samples were analyzed in duplicate.

For the relative quantification of gene expression, the $2^{-\Delta\Delta Ct}$ method developed by Weltzien et al. [62] was used applying the equation (1). The quantification of the expression of the gene of interest was expressed in relation to the expression of the selected reference gene. In addition, with the use of the calibrator that was included in all the plates, it was possible to minimize the differences in reaction performance that may exist between plates.

$$E = [2Ct\,(C) - Ct\,(M)] \times [2CtHK\,(M) - CtHK\,(C)] \quad (1)$$

where E is relative gene expression, C is the calibrator, M is the sample (gene of interest) and HK is the reference gene (*ELF-1α* and *β-actin*).

2.5. Statistical Analysis

Growth data, feed utilization and data obtained from histological parameters measurements were evaluated using a one-way analysis of variance (ANOVA), with initial live weight as covariate (Snedecor and Cochran, [63]). The Newman–Keuls test was used to evaluate specific differences between diets at a level of $p = 0.05$ (Statgraphics, Statistical Graphics System, Version Plus 5.1, Herndon, VA, USA).

To verify if there are significant differences in the gene expression between experimental groups, the Newman–Keuls test, a multivariate analysis of variance, was carried out, considering diet and different sections as factors: and the combination of both factors for each gene individually. The calculation was performed with a confidence interval of 95% ($p \leq 0.05$), and the data are shown as the mean ± SEM (standard error of the mean). Statistical data analyses were performed using the software Statgraphics© Centurion XVI (Statistical Graphics Corp., Rockville, MO, USA).

3. Results

3.1. Growth, Survival and Food Intake

Survival at the end of the experiment was 93.3, 81.7 and 51.1% in fish fed FM20, FM10 and FM0 diets, respectively. The mortality in all experimental groups was not progressive but was caused by a punctual increase in nitrite levels at the end of the experiment (day 70 of the trial). As all experimental groups were reared under the same recirculation freshwater system, all fish were submitted equally to environmental conditions.

In addition, there were significant differences in growth, with a lower final weight and SGR for the fish fed with the FM0 diet (51.1 g and 1.73% day^{-1}). The feed intake (FI) and the feed conversion ratio (FCR) also varied according to the treatment, being higher for the FM0 diet (2.1 g for 100 g $fish^{-1}$ day^{-1}) (Table 3), and consequently registering the lowest protein efficiency ratio (PER).

Table 3. Effect of the different diets on growth and nutritive parameters in rainbow trout.

	Experimental diets			*p*-Value
	FM0	**FM10**	**FM20**	
Initial weight (g)	13.60 ± 0.23	13.43 ± 0.23	13.13 ± 0.23	0.3911
Final weight (g)	49.07 ^{b} ± 1.69	72.94 ^{a} ± 1.69	72.24 ^{a} ± 1.69	0.0001
Survival (%)	51.11 ^{b} ± 5.62	81.67 ^{a} ± 5.62	93.33 ^{a} ± 5.62	0.0046
SGR (% day^{-1}) [1]	1.73 ^{b}± 0.04	2.24 ^{a}± 0.04	2.21 ^{a}± 0.04	0.0043
FI (g 100 g fish^{-1} day^{-1}) [2]	2.10 ^{a} ± 0.06	1.85 ^{b} ± 0.06	1.79 ^{b} ± 0.06	0.0188
FCR [3]	1.52 ^{b} ± 0.06	1.09 ^{a} ± 0.06	1.01 ^{a} ± 0.06	0.0018
PER [4]	1.62 ^{b}± 0.09	2.31 ^{a}± 0.09	2.49 ^{a}± 0.09	0.0025

[1] SGR, specific growth rate = 100 × ln (final weight/initial weight)/days. [2] FI, feed intake = 100 × feed consumption (g)/average biomass (g) × days. [3] FCR, feed conversion ratio = feed consumption (g)/biomass gain (g). [4] PER, protein efficiency ratio = biomass gain (g)/protein intake (g). Means of triplicate groups. Values are presented as mean ± SEM (n = 3). Values in the same row with different superscript letters are significantly different ($p < 0.05$). Initial weight was considered as covariable for final weight and SGR.

3.2. Intestinal health status

3.2.1. Intestinal histology

The intestine measurement parameters are shown in Table 4. FM0 registered lower VT, LP and higher GC numbers at proximal intestine (PI) parameters, confirming this higher number of GC in the distal intestine (DI). No significant parameters were observed in the rest of the proximal or distal parameters.

3.2.2. Gene Expression

Both reference genes provided standard deviations lower than 1.00 [64]; therefore, we decided to use the geometric average of both genes as a normalization factor in the current study. The mean of both genes was 18.56 ± 1.42 (*β-actin*, Ct) and 18.15 ± 1.65 (*ELF-1α*, Ct). The results are shown in Supplementary Table S2.

The results of gene expression, after a multifactorial analysis, showed differences between the inflammatory genes *IL-8* and *IL-1β* and the immune system *IFN-γ* depending on the diet administered to the fish, but not depending on the region of the intestine in which said genes are expressed (Table 5). Likewise, if the combined effect is considered, significant differences can be observed in most of the genes at anterior section.

Table 4. Effect of the different diets on proximal and distal measurements in rainbow trout.

	Experimental diets			*p*-Value
	FM0	**FM10**	**FM20**	
Proximal intestine				
VL (µm)	782.8 ± 77.6	831.6 ± 56.0	715.2 ± 47.5	0.2850
VT (µm)	209.9 ^{b} ± 14.1	164.6 ^{a} ± 10.2	149.8 ^{a} ± 8.6	0.0026
LP (µm)	53.7 ^{b} ± 4.5	35.4 ^{a} ± 3.3	33.7 ^{a} ± 2.8	0.0012
GC	2.7 ^{a} ± 1.5	8.9 ^{b} ± 1.0	15.2 ^{c} ± 0.9	0.0000
SL (µm)	67.8 ± 5.3	55.3 ± 4.7	62.7 ± 4.6	0.2184
ML (µm)	152.7 ± 13.0	117.3 ± 11.6	140.6 ± 11.4	0.1214
SML (µm)	60.8 ± 5.0	59.3 ±4.5	57.5 ± 4.4	0.8834
Distal intestine				
VL (µm)	547.5 ± 134.3	754.2 ± 54.8	756.8 ± 60.0	0.3396
VT (µm)	135.91 ± 26.9	160.88 ± 11.1	168.4 ± 11.8	0.5404
LP (µm)	26.9 ± 3.7	31.0 ± 1.5	33.2 ± 1.6	0.2633
GC	2.1 ^{a} ± 2.8	9.4 ^{b} ± 1.1	14.0 ^{b} ± 1.2	0.0006
SL (µm)	70.9 ± 12.6	80.0 ± 7.0	67.3 ± 9.6	0.5428
ML (µm)	93.3 ± 15.8	110.1 ± 8.7	110.4 ± 11.9	0.6266
SML (µm)	66.2 ± 7.1	50.1 ± 3.9	56.3 ± 5.3	0.1437

VL, villi length; VT, villi thickness; LP, lamina propria; GC, goblet cells; SL, serous layer; ML, muscular layer; SML, submucous layer. Values are the mean ± SEM (n = 18). Different letters in the same line denote significant differences ($p < 0.05$).

Table 5. p-values obtained after multifactor analysis.

	IgT	***Transferrin***	***IFN-γ***	***IL-8***	***IL-1β***	***TGF-β***
Function	Immune system	Immune system	Immune system	Inflammatory	Inflammatory	Inflammatory
Section	0.1966	0.2457	0.7111	0.1358	0.201	0.4442
Diet	0.1905	0.2857	0.0042 *	0.0088 *	0.018 *	0.4428
Interaction (section × diet)						
FM0-Ant vs. FM10-Ant vs. FM20-Ant	0.2137	0.0608	0.0177 *	0.014 *	0.0271 *	0.2446
FM0-Pos vs. FM10-Pos vs. FM20-Pos	0.4101	0.6864	0.2198	0.0512	0.2386	0.9588
FM0-Ant vs. FM0-Pos	0.8031	0.8249	0.5844	0.2244	0.7244	0.6994
FM10-Ant vs. FM10-Pos	0.1086	0.3628	0.1074	0.1703	0.2292	0.3474
FM20-Ant vs. FM20-Pos	0.2350	0.0620	0.5153	0.6035	0.3455	0.1546

Values with asterisk indicate significant differences were found ($p < 0.05$).

The results of gene expression for *IgT, Transferrin, IFN-γ, IL-8, IL-1β* and *TGF-β* in different intestinal sections and experimental diets are shown in Figure 1. The FM0 diet

induced, in general, higher expression, especially in anterior section. Fish belonging to the same diet group did not register differences between sections.

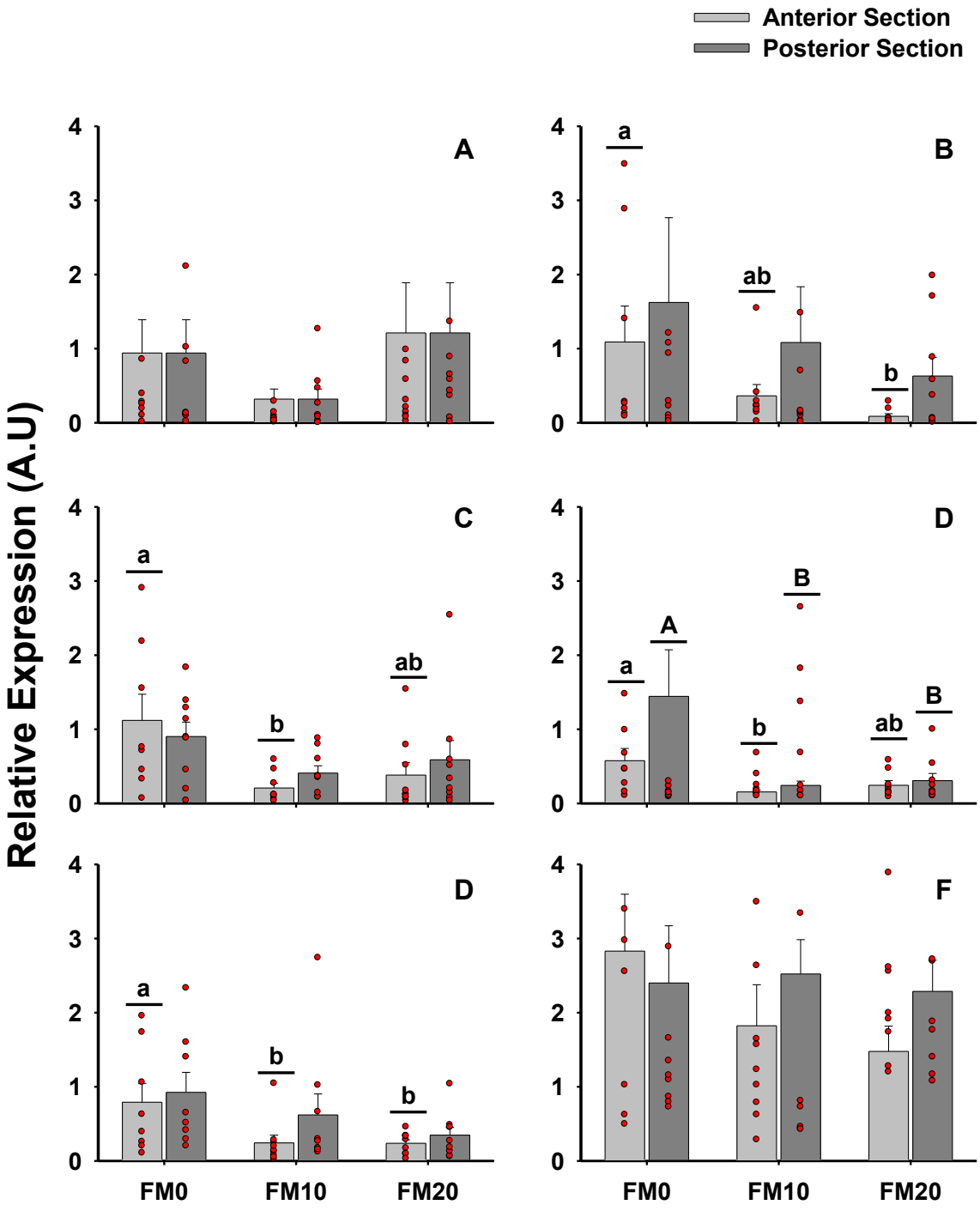


Figure 1. Gene expression of (**A**) *IgT*; (**B**)*Transferrin*; (**C**) *IFN-γ*; (**D**) *IL-8*; (**E**) *IL-1β* and (**F**) *TGF-β* genes taking into consideration intestinal section (anterior and posterior) and diet (FM0, FM10 and FM20). Lowercase letters reflect significant differences ($p < 0.05$) between diets in anterior section and capital letters reflect significant differences in posterior section.

Considering only the intestinal section, no significant differences were observed (Supplementary Figure S1). However, the registered average was usually higher in the posterior section with respect to the anterior section for all the genes studied. As no relevant results were observed between intestinal sections, from this point on the analysis was performed without considering this factor.

Regarding diet, regardless of the intestinal section (Supplementary Figure S2), the fish fed the FM0 diet presented higher values than those fed the other diets. Significant differences were found in the expression of the *IFN-γ, IL-8* and *IL-1β* genes. Finally, independently of the diet, *TGF-β* showed higher levels than the rest of the target genes (Supplementary Figure S3). No differences were found in the rest of the target genes.

4. Discussion

Although a total FM replacement was not possible without a diminishment in growth and fish health, a higher FM substitution (90%) than in previous trials [36,37] was achieved, maintaining good intestinal health and productive performance.

The SBM and WG combination was adequate to meet fish nutritional requirements without affecting fish growth, possibly due to the fact that the diet contained sufficient AA for fish growth. Despite the feed supplementation with synthetic AA to cover trout needs, especially in the plant-based diet, often the availability of these differs depending on diet composition, such as the amount of fiber and ANFs. Lower AA availabilities may lead to growth deficiencies or health status, as occurs with a lack of methionine, lysine and/or cysteine [65], due to their relevance in protein synthesis in relation to immune system, such as cytokines [66]. Therefore, this possible lower availability could have induced the higher intake in the FM0 diet. In previous studies the high inclusion of vegetable sources decreased the food intake, mainly due to the diet palatability; however, in the present work it seems that WG and SBM did not affect the palatability, and the higher intake could be attributed to the need to satisfy their requirements in essential AA. Therefore, the FM0 diet possibly presented an inadequate AA profile, which was confirmed by the PER results, which were significantly lower in the FM0 experimental group, which showed the worst protein quality and therefore an unbalanced AA profile.

As in previous studies [2,5,9,34,35], a total FM replacement was not feasible due to the lower growth performance results and the induction of immune and inflammatory responses at intestinal levels. Other studies on trout achieved good results with much lower FM substitution levels, using PP such as fermented SBM (40%) [67], sunflower meal or SBM (40%) [68], enzyme-treated SBM powder (50%) [69] and a mixture of CG, WG, extruded peas and rapeseed meal (50%) [70]. Higher substitutions (around 20%) have been successfully achieved in this species [9,10,37], and FM inclusion is currently accepted for rainbow trout, even in commercial feeds, with 16% of FM inclusion [71]. Generally, levels of plant meals in salmon and trout feed formulations are limited by their composition (relatively low CP and high crude fiber content) and by the presence of ANFs and non-soluble carbohydrates [46,72]. Nowadays, trout feed has lower carbohydrate levels, higher digestibility and higher energy levels (e.g., 34–55% CP, 18–32% fat, 10–20% starch) [73]. Commercial diet formulations have achieved low FM content, up to 16% [71], using a combination of alternative protein sources and synthetic AA to better balance the AA levels [74]. An example is Tau, a synthetic AA included in the study. Tau is a determinant AA in some carnivorous species [75]. Rainbow trout have the capacity to synthesize Tau from cysteine (Cys) [76]; however, the rate of synthesis may be inadequate to fulfill the Tau needs of rainbow trout fed an all-plant protein diet [77]. Gaylord et al. [78] conducted a trial with plant diets supplemented with Tau, observing that Tau supplementation in an all-plant protein diet appeared critical for maintaining growth rates and FCRs equivalent to fish fed fishmeal-containing diets. In fact, supplementation with a 5 g kg^{-1} diet of Tau in a plant-based diet was sufficient to increase growth to levels equivalent to those seen in fish fed FM diets.

In our study, the results obtained clearly indicate that up to 90% of FM (FM10) can be substituted without presenting high mortality, an inflammatory process, or compromising the growth performance. The high mortality was registered after an accidental increase in the level of nitrite at the end of the experiment. This increase in nitrites was consistent across all the tanks; therefore, it affected all experimental groups equally, so differences in eventuality may be attributed to an imbalance in diet, intestinal inflammation or an altered immune system.

It has been shown that PP sources can induce intestinal inflammation because many of them contain various ANFs, especially legumes [72]. The negative effect of SBM on

growth has been attributed to ANFs such as protease inhibitors, tannins, lectins and non-starch polysaccharides [79]. In the current study, the higher VT and LP values at PI were registered for the FM0 experimental group, probably due to the high content of these ANFs. The opposite trend seems to be registered at DI, lower VT and LP values, but without significant differences.

Similar inflammatory responses against FM substitution with plant diet has also been reported in salmonids [80], due to the infiltration of inflammatory cells, such as lymphocytes, macrophages, eosinophils and neutrophil granular cells, and diffuse immunoglobulin M (IgM). At FM replacement levels greater than 50%, rainbow trout and Atlantic salmon fed SBM-containing diets exhibited a progressive decline in growth rate, which was accompanied by a corresponding depression of non-specific immune capacity and exacerbation of pathological changes in the DI [11,18,81,82]. SBM levels in diets of 400–450 g kg^{-1} seem to be the inclusion limit, beyond which intestinal damage occurs in trout [3,40]; this can explain the VT of proximal intestine differences and the relatively higher surface in the fish fed the FM0 diet, for which SBM was included at 400 g kg^{-1}.

Furthermore, in the present study a lower GC number was found in both PI and DI of fish fed FM0 diet. A reduction in GC might denote an immunosuppression in rainbow trout fed diets without FM, such has been shown in previous studies carried out on rainbow trout fed PP for 63 days [83]. Goblet cells maintain the epithelial homeostasis through the secretion of a mucosal barrier that acts as a lubricant, preserving the epithelium, although some studies support that GC can act as a major cellular component of the innate and adaptative defense system [84]. In addition, a lower number of GC was also observed in other species such as gilthead seabream when fed with diets comprising 100% PP sources in substitution of FM or high PP levels [85]. The dietary effect on goblet cells may be caused by phytate or fibers contained in protein sources rather than other ANFs that can be eliminated by heat treatment [86].

In other studies, it has been proven that the use of SBM as a protein source also induces immune alterations. In Atlantic salmon (*Salmo salar*) the inclusion of 10% SBM produces detrimental inflammatory effects at the intestinal level [84], and a high dietary inclusion level of PPC (35%) resulted in significant adverse effects on growth performance, nutrient digestibility, digestive physiology and gut health [43]. High concentrations of dietary

soybean suppress salmonid growth rates and non-specific immune capacity. Burrells et al. [18] found that the immunosuppression became evident at protein soybean products inclusion rates of 60–70% in rainbow trout, which was coincident with a reduction in weight gains and the appearance of demonstrable pathological changes in the DI. Similarly, Jalili et al. [42] formulated diets for rainbow trout with different levels of FM substitution with vegetable protein sources (WG, CG and SBM) and according to their findings, higher PP inclusions (70 and 100%) resulted in undesirable effects on growth, nutritional indices, serum total immunoglobulin and alternative complement activity. Finally, they concluded that FM levels lower than 20% were not able to maintain growth rates and/or growth efficiencies with good health status [34,35,38].

Reinforcing the theory of altered intestinal health caused by a total FM substitution, higher expression of immune and inflammatory genes was reported in FM0 group, with high expressions of the genes *IL-1β, IL-8* and *IFN-γ*. This is consistent with the results observed in previous studies [25,87]. In particular, *IL-1β* and *IL-8* are two complementary pro-inflammatory cytokines, generally induced in the early stage of an immune response [88]. While *IL-1β* induces the growth and proliferation of T and B lymphocytes and macrophages [89], *IL-8* has chemotactic capacity and attracts neutrophils to the site of possible infection [90]. The production of *IL-8* is stimulated by the expression of *IL-1β*, so it is not surprising to see the simultaneous expression of these two cytokines and that similar patterns are exhibited. Regarding *IFN-γ*, it is also a cytokine of the immune system, whose role is the activation of macrophages, thus increasing their phagocytic capacity [57]. The increase in its gene expression in relation to the pro-inflammatory mediators *IL-1β* and *IL-8* has been reported as a mechanism of regulation of inflammation [87] and the activation of innate immunity in response to a possible infection [91]. Interestingly, they share the same expression pattern, which may also be because they are the same type of protein.

No significant differences were observed between the two sections for any of the genes studied. Unlike the present results, there are many other studies in which the posterior intestine is recognized as the site with the greatest inflammatory impact [3], and it is precisely the zone where there is a greater number of associated immune cells [92], and where the local immune response is usually higher [93]. In addition, the higher expression levels of the *TGF-β* gene in both sections, compared with the rest of the genes, could be

explained by the fact that this molecule fulfills a function both inhibiting and stimulating inflammation and, in addition to these antagonistic functions, it also exerts profound effects on immune cells, including lymphocytes and macrophages [94].

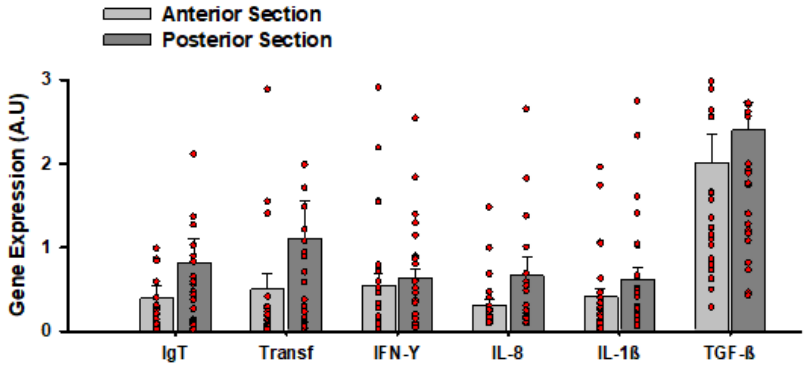
Considering the interaction between both factors, higher values were again recorded for the FM0 group, mainly in the anterior section, with the exception of *IgT* and *TGF-β*. This work shows opposite results to those observed in previous studies [87,95], in which the inclusion of vegetable proteins in the diet produced an increase in the expression of this specialized molecule in immune responses of the intestinal mucosa [56]. Regarding *Transferrin,* which is a plasma protein involved in iron supply, it creates an environment in the mucosa that is not suitable for bacterial survival when it binds to it. It functions as a response mechanism to the management of environmental stress and plays an important role in the innate immune system [96]. So, the higher level of PP inclusion in the FM0 group could be determining an intestinal inflammatory reaction that for a prolonged period may be responsible for the appearance of a certain local immunosuppression, causing an immune dysfunction, and, therefore, fish more susceptible to stress situations [25]. Thus, the alteration of inflammatory and immune intestinal status may have contributed to the lower survival of the FM0 group after the nitrite peak event.

Therefore, possible reasons for the lower growth and survival may be due to different factors or their combination. Besides the possible deficiency of some AA, the higher mortality observed in the FM0 group may be caused by the combination of histological intestinal alteration possibly induced by ANFs present in vegetable raw material, causing inflammation and maybe a certain level of immunosuppression [18,81].

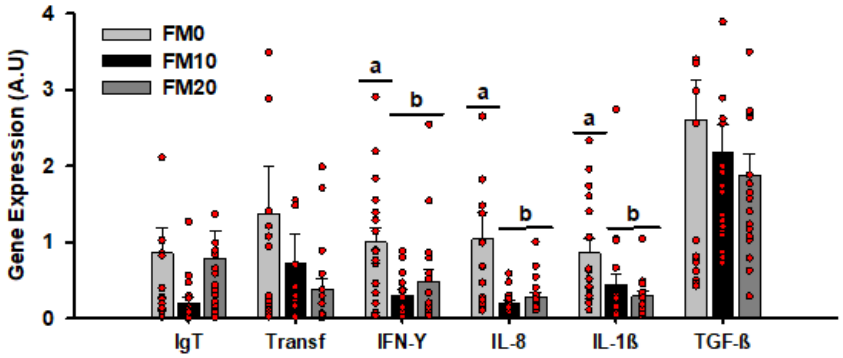
5. Conclusions

The results of this study show that 90% of FM substitution is feasible using a PP blend of WM, WG and SBM supplemented with Tau, Val, Lys and Met without apparent decrement in growth or intestinal health status.

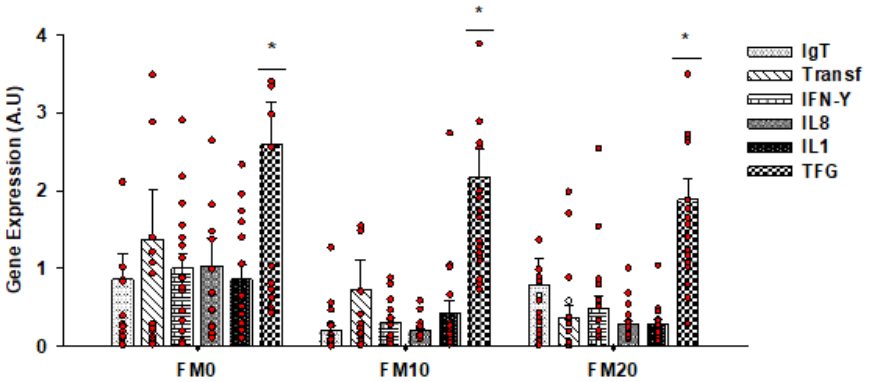
Supplementary Materials



Supplementary Figure S1: Gene expression of *IgT, Transferrin (Transf), IFN-γ, IL-8, IL-8, IL-1β* and *TGF-β* in anterior and posterior intestine section.



Supplementary Figure S2: Gene expression of *IgT, Transferrin (Transf), IFN-γ, IL-8, IL-1β* and *TGF-β* in each experimental diet: FM0, FM10 and FM20. Different letters reflect significant differences ($p < 0.05$).



Supplementary Figure S3: Gene expression of *IgT, Transferrin (Transf), IFN-γ, IL-8, IL-1β* and *TGF-β* based on experimental diet: FM0, FM10 and FM20. Different letters reflect significant differences ($p < 0.05$) in the expression.

Supplementary Table S1: Amino acid profile of experimental diets expressed in g per 100 g of wet matter.

	Experimental diets		
	FM0	**FM10**	**FM20**
Amino acids (% wet weight)			
EAA g 100 g^{-1}			
Histidine	1.32	0.87	1.04
Arginine	6.52	2.99	3.22
Valine	2.33	1.59	1.74
Methionine	0.70	0.67	0.54
Lysine	2.90	2.74	2.14
Isoleucine	1.88	1.35	1.47
Leucine	3.25	2.32	2.55
Phenylalanine	2.12	1.34	1.55
Threonine	1.79	1.22	1.41
NEAA g 100 g^{-1}			
Aspartic	4.12	3.08	3.13
Serine	2.77	1.79	1.96
Glutamic	13.94	9.77	9.53
Glycine	2.17	1.61	1.94
Alanine	1.75	1.42	1.60
Proline	4.60	3.36	3.53
Cysteine	0.65	0.73	0.53
Tyrosine	1.42	0.89	1.00

EAA, essential amino acids; NEAA, non-essential amino acids.

Supplementary Table S2: Results from BestKeeper program analysis.

	β-actin	***ELF-1α***
N	92	92
geo Mean [Ct]	18.54	18.12
Mean [Ct]	18.56	18.15
min [Ct]	17.30	15.41
max [Ct]	20.99	20.03
std dev [± Ct]	0.51	0.72
CV [% Ct]	2.74	3.99
min [x-fold]	-2.37	-6.54
max [x-fold]	5.45	3.75
std dev [± x-fold]	1.42	1.65
coeff. of corr. [r]	0.885	0.942

6. References

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CAPÍTULO 2

Effect of additives inclusion in gilthead seabream (*Sparus aurata* L.) diets on growth, enzyme activity, digestibility and gut histology fed with vegetable meals

Effect of additives inclusion in gilthead seabream (*Sparus aurata* L.) diets on growth, enzyme activity, digestibility and gut histology fed with vegetable meals

Glenda Vélez-Calabria, Ana Tomás-Vidal, David S. Peñaranda, Miguel Jover-Cerdá and Silvia Martínez Llorens

Aquaculture and Biodiversity Research Group. Institute of Science and Animal Technology. Universitat Politècnica de València, Valencia, Spain.

Animals 2023, *13*(2), 205

Abstract

The fishmeal replacement by vegetable meals or other alternative sources, without affecting fish performance and productivity, is one of the principal challenges in aquaculture. The use of hydrolyzed porcine mucosa (HPM) and nucleotide (NT) concentrates, as feed additives in gilthead seabream (*Sparus aurata* L.) non-fishmeal diets was assessed in order to determine the possible effects on growth, feed efficiency, protein digestion, and gut histology when these were included in a plant-based diet (HPM 1% and 2%, P1 and P2; NT 250 and 500 ppm, N250 and N500), in comparison with two control diets, AA0 (100% plant-protein-based diet) and FM100 (100% fishmeal-protein-based diet). Diets were assayed in triplicate and the growth assay lasted 134 days. Results showed a significant improvement in all groups in terms of final weight and specific growth rate in comparison with the AA0 group. An improvement in the feed conversion ratio and the protein efficiency ratio was also observed when the additives were included in lower percentages (P1 and N250) compared to the FM100 group. Significant differences were found in hepatosomatic index, villi thickness, and goblet cells. Thus, the inclusion of NT and HPM was tested as beneficial for the improvement of efficiency of plant feed in seabream.

Keywords: gilthead seabream, additives, hydrolyzed porcine mucosa, nucleotide concentrates

1. Introduction

The fishmeal replacement (FM) by alternative sources in diets for gilthead seabream (*Sparus aurata* L.) is necessary to ensure aquaculture sustainability. Many researchers have focused on finding new ingredients, such as a wide variety of vegetable meals, that can substitute FM in fish feed.

Good growth results have been obtained for gilthead seabream when 50% [1] and 75% [2] of the FM was replaced with mixtures of vegetable meals, although histological alterations in the gut were observed when plant ingredients were included in percentages above 60% [3], and also an immunosuppressive effect when the level of inclusion exceeded 75% [4]. Kissil and Lupatsch [5] replaced 100% of the FM by a mixture of high-quality vegetable sources and the addition of synthetic amino acids, but commercial application of the designed feed was unfeasible. Feed formulation with vegetable ingredients was limited by the protein content of different sources, the presence of antinutritional factors [6], and the balance of amino acids (AA).

Feed additives are added during feed preparation to improve not only the quality of the feed but also to improve the growth performance and health performance of the fish. Most feed additives are non-nutritive and include antioxidants, immunostimulants, probiotics, prebiotics, enzymes, microalgae, among others. Prebiotics are non-digestible feed ingredients that beneficially affect the host by selectively stimulating the growth or activity of one or a limited number of bacterial species already resident in the gut, and thus attempt to improve the host's health [7]. Prebiotics have been studied in fish since 1995 [8] and different effects, such as the increase in the growth rate, the stimulation of the immune system, changes in the gut microbiota, and modulation of the gut morphology have been described [9–13].

Nucleotides (NTs) are low-molecular-weight intracellular compounds which play key roles in nearly all biochemical processes [14] and are the building blocks of nucleic acid (DNA and RNA). A nucleotide consists of a nitrogenous base, a sugar (ribose or deoxyribose), and one to three phosphate groups. When the phosphate group of the NT is removed by hydrolysis, the structure remaining is nucleoside. Dietary supplementation of NTs or nucleosides has been shown to benefit many mammalian physiological and

nutritional functions [15–18] and both are considered as functional nutrients [19–21]. Although NTs can be synthesized endogenously from purines and pyrimidines originating from salvage (recycling from dead cells) and de novo synthesis (from AA) pathways in animals, it has been increasingly demonstrated that NT supplementation is required in diets for maximum production performance and immunocompetence of aquatic animals [22,23]. Moreover, (non-fishmeal) aquafeed ingredients contain relatively low amounts of nucleotides [24]. NTs participate in cell construction, tissue growth, development, and repair, fast growth, health benefits, and stress tolerance in fish, which have been regarded as "semi-essential nutritional components" or "conditional nutrition" [20,21]. NT concentrates (prebiotics) have been frequently tested in mammals, and beneficial effects on immune system [25], gut morphology [26], digestive microbiota [17], fat metabolism [27], and resistance to diseases [28] were observed many years ago. The roles of NTs and metabolites in fish diets have been sparingly studied for over 25 years [29]. NTs currently used in aquaculture [30,31] are the same as those added in infant formulae [32], including five types of nucleotide 5′-monophosphate at sodium salts, water soluble cytidine monophosphate, uridine monophosphate, AMP, inosine monophosphate (IMP), and GMP [33]. The addition of NT concentrates in feed has been tested in different species, such as rainbow trout (*Oncorhynchus mykiss*, [34]), Atlantic salmon (*Salmo salar*, [31]), red drum (*Sciaenops ocellatus*, [35]), Pacific white shrimp (*Litopenaeus vannamei*, [36]), meagre (*Argyrosomus regius*, [37]), red seabream (*Pagrus major*, [20]), and grass carp (*Ctenopharyngodon idellus*, [38]), with a variable effect on growth and survival rate. The information on the effect of NTs on feed efficiency, metabolism, and mitochondrial enzyme complexes for gilthead seabream is scarce. Couso et al. [39] described an increase in the survival rate of seabream juveniles fed with a feed which incorporated an NT concentrate, after they were exposed to the pathogenic bacteria *Photobacterium damselae* subsp. *piscicida*, which suggests an immunostimulant effect of the NT concentrate. El-Nokrashy et al. [40] found that dietary NTs supplemented at 250 mg/kg or 500 mg/kg enhanced the final body weight, weight gain, and specific growth rate of gilthead seabream, either with a dietary level of 25% FM diet or a non-fishmeal diet.

On the other hand, hydrolyzed porcine mucosa (HPM) is derived from the mucosa of the cleaned small intestine of pigs, and is a byproduct of the production of the anticoagulant drug heparin sodium after high-temperature spray drying [41]. To increase the yield of

heparin sodium, enzymes are added during the extraction process for the reaction [42]. Therefore, HPM contains polypeptides, oligopeptides, small peptides, and free AA [43,44]. As a new type of functional nutritive animal protein source, HPM has been widely used in lactating sows [45] and post weaning pigs [43,46,47] owing to its high protein content, balanced AA composition, and high safety profile [41,44]. There is little research on the use of HPM in aquatic animals. To the best of our knowledge, only one study on carp (*Cyprinus carpio*) has been reported thus far [48], which showed that replacing FM with 3% HPM equivalents had no significant effect on the growth performance of carp; however, there was a significant reduction in intestinal fold depth and villi height.

The current work assesses the effects of including, in vegetable feeds for fish, an NT concentrate and HPM in different doses. Growth, nutrient efficiency, biometric indices, dry matter, protein and AA digestibility, and intestinal histology have been evaluated and compared to values obtained in fish fed with FM.

2. Materials and methods

The experimental protocol was reviewed and approved by the Ethics and Animal Welfare Committee of the Universitat Politècnica de València (Official bulletin No. 80 of 06/2014), following Royal Decree 53/2013 and the European Directive 2010/63/EU on the protection of animals used for scientific research, with the purpose of minimizing the suffering of animals.

2.1. Rearing system

The trial lasted 134 days and was conducted in 18 cylindrical fiberglass tanks (1750 L) within a recirculating saltwater system (75 m^3 capacity) with a rotary mechanical filter and a gravity biofilter (6 m^3 capacity). All tanks were equipped with aeration, and the water was heated with a heat pump installed in the system. The water temperature was 22.0 ± 0.52 °C, salinity was 30 ± 1.7 g L^{-1}, dissolved oxygen was 6.5 ± 0.49 mg L^{-1}, and pH ranged from 7.5 to 8.5. The photoperiod was natural, and all tanks had similar lighting conditions.

2.2. Fish

Gilthead seabream were obtained from the fish farm PISCIMAR in Burriana (Valencia, Spain) and after two weeks of acclimation to laboratory conditions, fed a standard commercial diet (480 g kg^{-1} crude protein, CP; 230 g kg^{-1} crude lipid, CL; 110 g kg^{-1} ash; 22 g kg^{-1} crude fiber, CF and 140 g kg^{-1} nitrogen-free extract, NFE), and were distributed in 18 cylindrical fiberglass tanks (three per treatment) in groups of 25 fish in each tank. The average weight of fish was 11 ± 1.2 g at the initiation of the experiment.

2.3. Diets and feeding

Diets were prepared as pellets by extrusion cooking with a semi-industrial twin-screw extruder (CLEXTRAL BC-45, Firminy, St Etienne, France) located at the Universitat Politècnica de València. The processing conditions were as follows: a temperature of 110 °C, a pressure of 40–50 atm, and a screw speed of 100 rpm.

In the vegetable diet (AA0), the protein fraction was supplied entirely by commercial plant ingredients, complying with the minimum requirements of essential amino acids (EAA) established by Peres and Oliva-Teles [49]. Additives were assayed in different doses: nucleotide concentrate in doses of 250 ppm (N250) and 500 ppm (N500), and HPM at levels of inclusion of 1% (P1) and 2% (P2), replacing wheat gluten. All these diets were isonitrogenous and isoenergetic and had the same chemical composition. In the control diet (FM100), all the protein was provided by FM. An amount of 50 g kg^{-1} of chromium oxide (Cr_2O_3) was used as an inert marker for the digestibility trial in these same diets. All dry ingredients were uniformly mixed together before adding the liquid ingredients (vegetable and fish oils). Ingredient content is shown in Table 1.

Each experimental diet was assayed in three different tanks, randomly assigned. Fish were handfed three times per day (8:00, 12:00, and 16:00) to apparent satiation in a weekly feeding regimen of six days and one of fasting. Pellets were distributed slowly, permitting all fish to eat. Fish were observed daily in tanks and were weighed individually every four weeks, using clove oil containing 87% eugenol (Guinama®, Valencia, Spain) as an anesthetic (1 mg per 100 mL of water) to minimize their suffering, in order to evaluate fish growth along the assay, determine growth parameters, and assess their health status.

2.4. Proximate composition and amino acids analysis

Chemical analyses of the dietary ingredients were determined prior to diet formulation. Diets and their ingredients were analyzed according to AOAC [50] procedures: dry matter (105 °C to constant weight), ash (incinerated at 550 °C to constant weight), crude protein (N × 6.25) by the Kjeldahl method after an acid digestion (Kjeltec 2300 Auto Analyser, Tecator, Höganas, Sweden), crude lipid extracted with methyl-ether (Soxtec 1043 Extraction Unit, Tecator), and crude fiber by acid and basic digestion (Fibertec System M., 1020 Hot Extractor, Tecator). All analyses were performed in triplicate.

Table 1. Formulation of experimental diets.

	AA0	N250	N500	P1	P2	FM100
Ingredients (g kg^{-1})						
Fishmeal						589
Wheat meal						260
Wheat gluten	290	289.75	289.5	280	270	
Bean meal	44	44	44	44	44	
Soybean meal	182	182	182	182	182	
Pea meal	44	44	44	44	44	
Sunflower meal	181	181	181	181	181	
Fish oil	78	78	78	78	78	38
Soybean oil	78	78	78	78	78	93
Soy lecithin	10	10	10	10	10	10
Vitamin–mineral mix *	10	10	10	10	10	10
Calcium phosphate	38	38	38	38	38	
Taurine	20	20	20	20	20	
Methionine	7	7	7	7	7	
Lysine	10	10	10	10	10	
Arginine	5	5	5	5	5	
Threonine	3	3	3	3	3	
Nucleotide concentrates		0.25	0.5			
Hydrolyzed porcine mucosa				10	20	

* Vitamin and mineral mix (values are g kg^{-1} except those in parenthesis): Premix: 25; Choline, 10; DL-α-tocopherol, 5; ascorbic acid, 5; $(PO_4)_2Ca_3$, 5. Premix composition: retinol acetate, 1 000 000 IU kg^{-1}; calcipherol, 500 IU kg^{-1}; DL-α-tocopherol, 10; menadione sodium bisulphite, 0.8; thiamine hydrochloride, 2.3; riboflavin, 2.3; pyridoxine hydrochloride, 15; cyanocobalamine, 25; nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07; ascorbic acid, 75; inositol, 15; betaine, 100; polypeptides 12. Zn, 5; Se, 0.02; I, 0,5; Fe, 0.2; CuO, 15; Mg, 5.75; Co, 0.02; Met, 1.2; Cys, 0.8; Lys, 1.3; Arg, 0.6; Phe, 0.4; Trcp, 0.7; excpt. 1000 g; Dibaq-Diproteg.

Following the method previously described by Bosch et al. [51], AA of diets were analyzed through a Waters HPLC system (Waters 474, Waters, Milford, MA, USA) consisting of two pumps (Model 515, Waters), an auto sampler (Model 717, Waters), a fluorescence detector (Model 474, Waters), and a temperature control module. Aminobutyric acid was added as an internal standard pattern before hydrolyzation. The

AA were derivatized with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Methionine and cysteine were determined separately as methionine sulphone and cysteic acid after oxidation with performic acid. AA were separated with a C-18 reverse-phase column Waters Acc. Tag (150 mm × 3.9 mm), and then transformed to methionine and cystine. Proximate composition and EAA content of experimental diets is shown in Table 2.

Table 2. Proximate composition of experimental diets.

	Experimental Diets [1]	**FM100**
Proximate composition (% on dry matter)		
Dry matter (DM) [2]	94	88
Crude protein (CP)	45.3	45
Total carbohydrates (CHO) [3]	29.1	23.2
Crude lipid (CL)	20.1	20
Crude fiber (CF)	2.5	1.5
Ash (A)	6.8	10.3
Crude energy (CE) (MJ Kg^{-1}) [4]	17,180	18,827
CP/CE (g MJ^{-1})	38.0	41.9
Essential amino acids (% on dry matter)		
Arginine (Arg)	2.68	3.55
Histidine (His)	0.94	1.57
Isoleucine (Ile)	1.67	2.09
Leucine (Leu)	3.06	4.07
Lysine (Lys)	2.40	3.64
Methionine (Met)	1.33	1.41
Threonine (Thr)	1.58	2.17
Valine (Val)	1.77	2.41

[1] Experimental diets (AA0, N250, N500, P1, and P2) were considered chemically identical. [2] Dry matter was expressed as % of wet matter. [3] Total carbohydrates were calculated as: CHO = 100 – CP – CL – CF – A. [4] Calculated using: 23.9 kJ g^{-1} protein, 39.8 kJ g^{-1} lipid and 17.6 kJ g^{-1} carbohydrate.

2.5. Growth and nutrient efficiency indices

The growth and nutrient efficiency indices were determined at the end of the experiment and the tank was used as an experimental unit. The specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), protein efficiency ratio (PER), and survival (S) were obtained, taking into account the monthly reported biomass of dead fish.

2.6. Biometric indices

At the end of the feeding trial, all the fish were individually weighed. Three fish from each tank, nine per treatment, were randomly slaughtered using a lethal bath of clove oil (150 mg L^{-1}), for the determination of biometric indices. The samples from each tank

were pooled and stored at −30°C. Fish total weight and length, as well as viscera, visceral fat, and liver weights were recorded for determination of condition factor (CF), viscerosomatic (VSI), visceral fat (VFI), and hepatosomatic (HSI) indices.

2.7. Digestibility

Apparent digestibility experiment was carried out in the same tanks at the end of the growth experiment. The same six diets were used but chromium oxide (50 g kg^{-1}) was added as an inert marker. The fish that were not used for the analyses were left in the tanks and continued to feed with these diets. Feces collection was carried out three times a week (to minimize fish suffering) for a period of 21 days. Fish were fed once a day in the morning (05:00) and fecal collection took place 8 h later (13:00). Before feeding, fish were fasted for two days. Extraction was performed by stripping (applying pressure on the ventral region from the pelvic fins to the anus). Wet fecal content was collected and dried at 60 °C for 48 h prior to analysis.

Fecal composition of DM, CP, and AA was analyzed by the same procedure as in diets, after drying at 100 °C until constant weight. Chromium oxide was determined in the diets and feces using an atomic absorption spectrometer (Perkin Elmer 3300, Perkin Elmer, Boston, MA, USA) after acid digestion. Analysis was performed in duplicate. DM, CP, and AA of diet and feces were used to determine apparent digestibility coefficient (ADC) of DM, CP, and AA with the following formula:

$$\text{ADC (\%)} = 100 \times [1 - (\text{\% marker in diet}/\text{\% marker in feces}) \times (\text{\% N in feces}/\text{\% N in diet})] \quad (2)$$

where N is the nutrient (DM, CP, and AA).

2.8. Histological analysis

Histological analysis of the current study was performed on fish fed with the control diet AA0 (diet to improve), FM100 (diet control), and the diets with which the best results were obtained, P1 and N250. At the end of the growth experiment and before the histological analysis, the guts of three fishes per tank fed with FM100, AA0, N250, and P1 diets were divided in three portions: proximal intestine (PI), middle intestine (MI), and distal intestine (DI). PI samples were preserved in phosphate-buffered formalin (4%, pH 7.4). All of the formalin fixed tissues were routinely dehydrated in ethanol, equilibrated

in ultraclean, and embedded in paraffin according to standard histological techniques. Transverse sections were cut with a thickness of 5 µm with a microtome Shandon Hypercut (five sections per paraffin block were obtained) and stained with haematoxylin and VOF (Light green, Orange G, and Fuchsin) for examination.

A total of 108 sections were analyzed under the light microscope (Eclipse E400 Nikon, Izasa S.A., Barcelona, Spain). For the measurements and observations of the intestine, we used a combination of criteria reported by several authors [4,52,53]. Daprà et al. [54] and Øverland et al. [55] use the following parameters: serous layer (SL), muscular layer (ML), submucous layer (SML), villi length (VL), villi thickness (VT), and lamina propria (LP). Six measurements per section of each parameter were performed, and average values were determined. In addition to the measurements, a quantification of goblet cells (GC) was made by counting the number of GC present in each villus. We used six villi per section.

2.9. Digestive enzyme activity

Digestive tracts of three fish per tank were sampled at the end of the assay, 134 days after initiation of the experiment. To ensure the presence of content along the whole digestive tract, fish were fed at 20:00 on the day before and at 8:00 on the sampling day.

Fish were dissected in order to obtain the digestive tract, after being anesthetized using clove oil and sacrificed by cold shock. Two different kinds of samples were considered and obtained: stomach (S) and gut (G). They were stored at −20 °C until enzymatic extraction. Enzyme extracts for protease analysis were obtained by manual disaggregation, dilution in distilled water (1 g of sample: 3 mL of distilled water), followed by homogenization by T25 digital ULTRA-TURRAX® (IKA®, Staufen, Germany), maintaining tubes on ice, and centrifugation at 12,000 rpm and 4 °C for 15 min. Supernatant were stored at −20 °C until enzyme analysis.

Pepsin assays were performed on S samples and total alkaline protease, trypsin, α-amylase, and alkaline phosphatase (ALP) assays were performed on G. Enzyme activities, expressed as U per g of tissue.

Acid protease (pepsin) activity was evaluated using 0.5% hemoglobin *w/v* as substrate in 100 mM glycine—HCl buffer, pH 2.5, at 280 nm, following the method detailed by

Anson [56] and modified by Díaz-López et al. [57]. One unit of activity was defined as 1 μg of tyrosine released per min (extinction coefficient = 0.0071 mL μg^{-1} cm^{-1}).

Trypsin activity was obtained by a kinetic assay using Nα-Benzoyl-DL-arginine p-nitroanilide (0.5 mM BAPNA) as a substrate in 50 mM Tris-HCl buffer containing 20 mM $CaCl_2$, pH 8.2, following the method developed by Erlanger et al. [58]. The increase in absorbance at 405 nm was measured every 30 s for 5 min. One unit of activity was defined as 1 μg of p-nitroanilide released per min (extinction coefficient = 0.0637 mL μg^{-1} cm^{-1}).

Total alkaline protease activity was tested using 1% casein *w/v* as substrate in 100 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 7.5, at 280 nm, following the method detailed by Kunitz [59] and modified by Walter [60]. One unit of activity was defined as 1 μg of tyrosine released per min (extinction coefficient = 0.0071 mL μg^{-1} cm^{-1}).

α-Amylase activity was determined by a kinetic assay using a commercial kit (Amylase MR, Cromatest, Linear Chemicals S.L., Barcelona, Spain), following manufacturer's instructions. The increase in absorbance at 405 nm was measured every 30 s for 5 min, after an incubation period of 1 min. One unit of activity was defined as 1 μg of 2-chloro-p-nitrophenol released per min during the enzymatic reaction at 37 °C (Extinction coefficient = 0.0818 mL μg^{-1} cm^{-1}).

The samples for alkaline phosphatase (ALP) were diluted at a ratio of 1:20. The activity of this enzyme was measured using a kinetic assay commercial kit (ALP-LQ, Spinreact, liquid, DGKC, St Esteve d'en Bas, Girona, Spain), following manufacturer's instructions. A total of 200 μL of reagent were added to 10 μL of the diluted homogenate. The increase in absorbance at 405 nm was measured every 30 s for 5 min, after an incubation period of 1 min. One unit of activity was defined as 1 μg of p-nitrophenylphosphate released per min during the enzymatic reaction at 25 °C.

2.10. Statistical analysis

The results of the different growth and nutrient indices, biometric indices, ADCs, histological measurements, and specific enzyme activities were analyzed through an analysis of variance using the statistical package Statgraphics® Plus 5.1 (Statistical

Graphics Corp., Rockville, MO, USA), with a Newman–Keuls test for the comparison of the means. Initial weight was used as a covariate in the analysis of growth indices. The results are shown as the mean ± standard error (SEM). The level of significance was set at $p < 0.05$.

3. Results

3.1. Growth and nutrient efficiency indices

The results obtained on growth and nutrient efficiency indices are shown in Table 3. At the end of the growth period, all groups showed a significant improvement in final body weight and specific growth rate (SGR) in comparison with the control group (AA0), which showed the lowest values (87 g and 1.59% day^{-1}, respectively), although slightly inferior to the FM100 diet. All diets were well accepted and no significant statistical differences between groups for feed intake (FI) and survival rate (S) were detected. The feed conversion ratio (FCR) was significantly better when the additives were added in lower percentages (P1 and N250), without significant differences compared to the FM100 diet. Similarly, the protein efficiency ratio (PER) presented values equal to and/or close to the FM100 diet when the additives were included in lower levels.

Table 3. Growth and nutritive efficiency indices of gilthead seabream fed the different experimental diets.

	AA0	N250	N500	P1	P2	FM100	SEM
Initial weight (g)	13 ^{a}	11 ^{ab}	11 ^{ab}	10 ^{b}	10 ^{b}	9.5 ^{b}	0.8
Final weight (g)	87 ^{e}	111 ^{c}	107 ^{c}	121 ^{b}	99 ^{d}	152 ^{a}	1.4
SGR (% day^{-1}) [1]	1.59 ^{e}	1.74 ^{c}	1.71 ^{c}	1.81 ^{b}	1.65 ^{d}	2.09 ^{a}	0.01
FI (g 100 g fish^{-1} day^{-1}) [2]	2.14	1.96	2.01	1.87	2.05	2.04	0.10
FCR [3]	1.93 ^{b}	1.60 ^{a}	1.66 ^{ab}	1.50 ^{a}	1.70 ^{ab}	1.57 ^{a}	0.11
PER [4]	1.24	1.38	1.32	1.41	1.28	1.41	0.08
S (%) [5]	89.1	77.3	85.1	84.7	79.6	85.5	4.95

[1] Specific growth rate (SGR, % day^{-1}) = 100 × ln (final weight/initial weight)/days. [2] Feed intake (FI, g 100 g fish^{-1} day^{-1}) = 100 × feed consumption (g)/average biomass (g) × days. [3] Feed conversion ratio (FCR) = feed offered (g)/weight gain (g). [4] Protein efficiency ratio (PER) = weight gain (g)/protein offered (g). [5] Survival (S, %) = 100 × (final number of fish/initial number of fish). Data are presented as mean ± SEM ($n = 3$). Different superscript letters indicate significant differences amongst treatments ($p < 0.05$).

3.2. Biometric indices

The values obtained in the biometric indices are shown in Table 4. No significant differences were found in CF, VSI, and VFI biometric indices, with the exception of the hepatosomatic index (HSI), which in the N500 group was the lowest value.

Table 4. Biometric indices of seabream fed with different experimental diets.

	AA0	N250	N500	P1	P2	FM100	SEM
CF (g cm^{-3}) [1]	1.60	1.65	1.61	1.67	1.55	1.66	0.04
VSI (%) [2]	12.01	11.93	12.40	12.68	11.43	12.52	0.97
VFI (%) [3]	0.61	0.75	0.64	0.59	0.64	0.70	0.16
HSI (%) [4]	1.79 ^{ab}	1.69 ^{ab}	1.60 ^{b}	1.69 ^{ab}	1.68 ^{ab}	1.99 ^{a}	1.68

[1] Condition factor (CF, g cm^{-3}) = 100 × total weight (g)/total length^3 (cm). [2] Viscerosomatic index (VSI, %) = 100 × visceral weight (g)/fish weight (g). [3] Visceral fat index (VFI, %) = 100 × visceral fat weight (g)/fish weight (g). [4] Hepatosomatic index (HSI, %) = 100 × liver weight (g)/fish weight (g). Data are presented as least-squares means ± SEM (n = 9). Different superscript letters indicate significant differences amongst treatments ($p < 0.05$).

3.3. Digestibility

Apparent digestibility coefficients (ADC) of dry matter (ADC_{DM}), crude protein (ADC_{CP}), and amino acids (ADC_{AA}) of the diets for gilthead seabream are presented in Table 5. The ADC results obtained were similar in all treatments, not appreciating differences in digestibility due to the use of additives compared to the non-fishmeal control diet (AA0).

Table 5. Apparent digestibility coefficients (ADC, %) of crude protein (CP), dry matter (DM), and amino acids (AA) in the gilthead seabream fed different experimental diets.

	AA0	N250	N500	P1	P2	FM100
ADC_{CP}	91	93	93	91	93	88
ADC_{DM}	63	61	62	73	70	81
ADC_{EAA} [1]						
Arg	89	90	88	93	90	96
His	93	95	95	95	93	96
Ile	92	94	94	95	92	96
Leu	93	96	95	95	93	96
Lys	94	95	96	96	94	98
Met	96	98	98	98	97	97
Phe	95	96	96	97	95	97
Thr	91	93	93	94	90	96
Val	91	94	94	94	91	96
ADC_{NEAA} [2]						
Ala	91	92	93	94	90	96
Asp	87	91	92	91	86	92
Aba	89	92	92	93	89	92

Glu	96	97	97	97	96	97
Gly	88	90	90	91	86	93
Pro	95	96	96	96	95	96
Ser	93	95	94	95	93	96
Tyr	95	97	97	97	96	98
AADCEA [3]	93	95	94	95	93	97

[1] EAA, essential amino acids; [2] NEAA, non-essential amino acids; [3] AADCEA, average apparent digestibility coefficient of essential amino acids.

3.4. Histological analysis

The results of the measurement of each morphological parameter evaluated in the PI are shown in Table 6.

Table 6. Effect of the different experimental diets on proximal intestine parameters.

	AA0	**N250**	**P1**	**FM100**	**SEM**
SL (µm)	34.1	36.5	37.2	34.5	1.4
ML (µm)	35.1	35.1	37.5	36.6	1.3
SML (µm)	27.1	23.8	25.3	24.8	1.6
VL (µm)	513.9	470.0	514.7	482.7	34.3
VT (µm)	101.2 [a]	102.9 [a]	110.8 [ab]	114.7 [b]	4.1
LP (µm)	20.4	21.1	21.2	20.8	1.0
GC	8.5 [b]	7.6 [ab]	7.8 [ab]	6.9 [a]	1.1

Serous layer (µm), SL; Muscular layer (µm), ML; Submucous layer (µm), SML; Villi length (µm), VL; Villi thickness (µm), VT; Lamina propria (µm), LP; Goblet cells, GC. Data are mean ± SEM (n = 9). Different letters indicate that significant differences were observed ($p < 0.05$).

3.5. Digestive enzyme activity

Activities of digestive enzymes pepsin and α-amylase were unaffected by diets composition, unlike total alkaline proteases, trypsin and alkaline phosphatase, which were affected. Fish fed the control diet AA0 had the lowest enzymatic activity values, except for α-amylase (Figure 1).

3.5.1. Proteases activity

No significant differences were found in pepsin activity in stomach samples from fish fed the different experimental diets (Figure 1a). The highest value was obtained in P1 group, followed by N250, while the activity registered in the other groups was lower. Total alkaline proteases activity showed significant differences in the gut tissue (Figure 1b). The control diets (AA0 and FM100) showed differences from each other, while the groups with additives (N250, P1, N500, and P2) did not, presenting very close values. Significant differences in trypsin activity were found, where fish fed the P2 diet showed the highest values, followed by N250, P1, N500, and AA0 diets (Figure 1c).

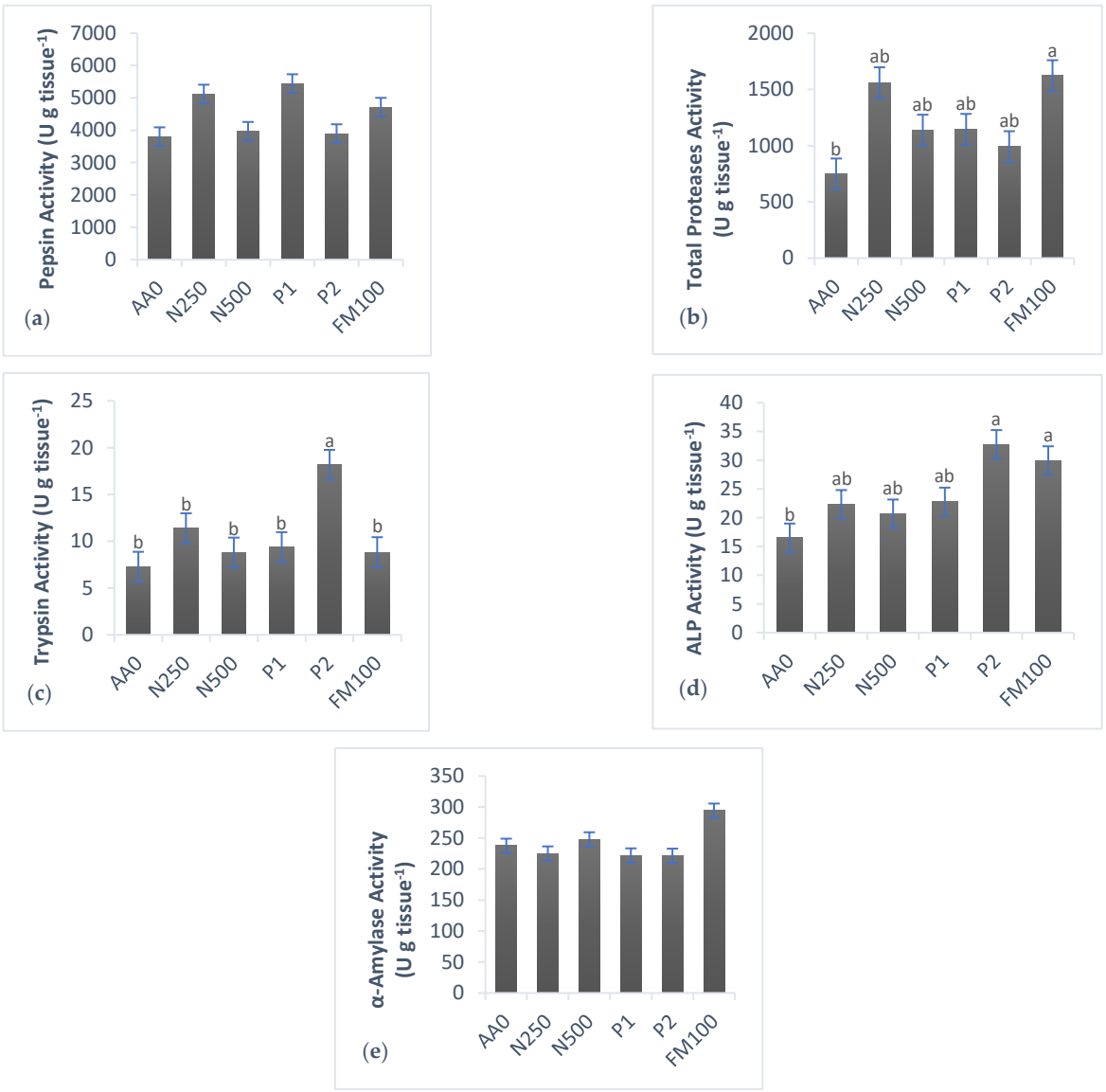

Figure 1. Digestive enzyme activity expressed in U g tissue^{-1} and determined in the gastrointestinal tissue of fish fed the different experimental diets. (**a**) Pepsin activity in stomach tissue; (**b**) total alkaline proteases activity in gut tissue; (**c**) trypsin activity in gut tissue; (**d**) alkaline phosphatase (ALP) activity in gut tissue; (**e**) α-amylase activity in gut tissue. Means of three fish per treatment ± SEM (n = 9). Different superscripts indicated differences ($p < 0.05$).

3.5.2. Alkaline phosphatase activity

There were significant differences in ALP (brush border enzyme) activity. As in the activity of the total alkaline proteases, the control diets AA0 and FM100 presented differences between them. The groups with additives (P1, N250, and N500) showed similar values, with the P2 group presenting the highest value (Figure 1d).

3.5.3. α-Amylase activity

There were no significant differences observed among experimental groups when α-amylase activity was determined in gut tissue (Figure 1e). Highest average values were registered in the N500 group followed by the AA0 control group.

4. Discussion

The results in the present study clearly show that the inclusion of additives (HPM and NT concentrate) to a 100% vegetable diet, improves the values in all groups significantly in terms of growth performance and nutrient efficiency indices, compared to the control group (AA0). When the additives were included in low concentrations, the P1 and N250 groups showed the highest results regarding the final weight, SGR, FCR, and PER. Similarly, the N500 and P2 groups showed close values. Regarding the HPM or HPM equivalents, Yang et al. [61] found the addition of low doses of enzyme-digested hydrolyzed porcine mucosa (30 g kg^{-1}) feasible in hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) feed, as there were no significant changes in growth performance in terms of SGR, PER, and FCR compared with controls. Likewise, results obtained by Gao et al. [48] suggest that dried porcine soluble, a byproduct of heparin extraction from pig intestines, can replace FM without any adverse impact on the growth performance of carp (*C. carpio*), thus confirming that additives greatly improve the use of vegetable diets. This may be due to the fact that the HPM has a high level of free AA and oligopeptides, which gives it high digestibility. With reference to NT concentrate, El-Nokrashy et al. [40] found that dietary NTs supplemented at 250 mg kg^{-1} or 500 mg kg^{-1} enhanced the final body weight, weight gain, and SGR of gilthead seabream compared to the control group. In contrast, the results obtained by Ridwanudin et al. [62] clearly showed that there was no positive effect of dietary nucleotides on fish growth of juvenile rainbow trout (*Oncorhynchus mykiss*). Commercial NT concentrates are normally a balanced concentrate of free NTs and active precursors, obtained from yeast. Thanks to these characteristics, they minimize the intestinal inflammatory response in diets with high incorporation of raw materials of plant origin, helping to enhance the digestibility of the diet and thus improve the intestinal health of animals. Generally, nucleoproteins are degraded by proteases to peptides and nucleic acids. The nucleic acids are cleaved by nucleases to NT. The phosphate groups of NTs are removed primarily by intestinal

alkaline phosphatases to form nucleosides. Intestinal proteases and alkaline phosphatases are well elucidated in fishes. Sugars may be cleaved by nucleosidases to produce free purine and pyrimidine bases. The nucleosides and nitrogenous bases are absorbed by the gut mucosa. The digestion and absorption of NTs in fish are influenced by various environmental and/or physiological factors [63].

In the present study, no significant differences were found in CF, which is supported by the results obtained by Gao et al. [48] and Yang et al. [61] when they replaced FM with HPM equivalents in diets for carp and hybrid grouper, respectively. The VSI did not show significant differences either, such as in the study by Ridwanudin et al. [62], in diets with dietary NT for rainbow trout, unlike Gao et al. [48] and Yang et al. [61], who did find that the VSI increased significantly in diets that included HPM equivalents compared with the control group. The HSI presented significant differences, where diets N250, P1, and P2 showed values close to the control diet. This may be due to the direct utilization of the content of small peptides present in the feed, which increases metabolic load and liver mass [64]. The HSI of the N500 diet was significantly lower than the control diet. Similarly, Ridwanudin et al. [62] found that the effect of dietary NT significantly decreased HSI in rainbow trout. Pafundo et al. [65] found that hepatocyte size decreased in rainbow trout after NT treatment. Thus, increased NT availability in rainbow trout appeared to decrease hepatocyte size and ultimately reduced HSI.

The digestibility results obtained in the present trial were similar in all treatments, with no differences in digestibility due to the use of feed-additive NT concentrate and HPM, compared to the fishmeal-free diet (AA0). Fish fed with diets containing high concentrations of plant protein (PP) often show low feed digestibility, as a consequence of using ingredients known to be high in fiber or have very high starch content [66,67]. Supplementation of feeding stimulant, which helps to increase the digestibility of plant protein-based diet through increasing the secretion of various digestives enzymes, is also well documented [68–70]. The results obtained by Hossain et al. [23,71] indicate that supplementation with IMP nucleotide increased the efficiency of using soy protein concentrate (SPC $\leq$ 75%) as the only source of protein, and that NT supplementation may be effective as a functional supplement in the diet of red seabream (*Pagrus major*). The absorption of hydrolyzed proteins can be three times faster than those that are not

hydrolyzed [72]. The intestines of animals can absorb AA easier when they are in the form of small peptides, thus the incorporation of hydrolyzed proteins in diets can benefit the growth and development of animals [73], since the hydrolysis process transforms longer protein chains into smaller peptide chains and free AA. The results of the trial carried out by dos Santos Cardoso et al. [74] showed that the hydrolyzed swine mucus protein (HSMP) used at 20% in Nile tilapia (*Oreochromis niloticus*) diets has higher ADCs of protein, energy, and various AA.

In this study, it can be seen that the histological parameters SL, ML, SML, VL, and LP of PI were not significantly affected by diet, unlike VT and GC, which did present significant differences. Digestive functioning depends on the development of the intestine; better development of intestinal villus length and villus width increases the intestinal surface area for better nutrient absorption [75]. Our results indicate that in the P1 group, the VL and VT were numerically higher than that of the control group (AA0). This may be because small peptides can effectively stimulate and induce increases in brush border (BB) enzyme activity in the intestinal chorion and accelerate villus growth, promoting intestinal digestive function [76].

No significant differences were found in the LP, although the non-fishmeal diet (AA0) reached the lowest value of all. In several studies, the histological results show a circulatory disorder, with a marked infiltration of leukocytes and eosinophilic cells in the LP, when using diets with maximum replacement that imply an increase in the cellularity of the LP [53,55,77,78].

Considering the SL and ML, these did not present significant differences, resulting-in the smallest thickness in both parameters, obtained in the group of the control diet (AA0) with respect to the other diets. In contrast, the results obtained by Yang et al. [61] in hybrid grouper feed indicate that the muscle thickness of PI in control group was significantly higher than in the groups with HPM equivalents addition. Normally, SL is the layer located on the outside of the ML, it is formed by a secretory epithelial layer (mesothelium) and another layer of connective tissue that supplies nutrients to the epithelium through blood vessels [79]. The ML is the thinnest part of the digestive tract in the distal portion. The ML is the area where much of the nutrient uptake occurs, but virtually no mechanical processing occurs [80].

The SML no showed significant differences in the different experimental diets, the N250 diet being the lowest. Dietary NTs are a group of additives that are widely used in aquaculture as feed attractants. They are often implicated in numerous positive physiological effects including increased growth performance, feed utilization, and enhanced intestinal fold morphology in several fish and shellfish species [23,31,81]. In addition, NTs were shown to have a protective effect in overcoming intestinal and inflammatory reactions induced by plant-rich protein diets [37]. SML is a layer composed of connective tissue, that controls the expansion of the intestine when substantial food is consumed in carnivorous fish [82], and is also responsible for most of the absorption. It has numerous blood vessels, which make it possible to allow oxygen and nutrients to reach all the cells and also remove waste material [83].

Regarding the number of CG, significant differences were observed, and their number was lower in the N250 diet compared to the control diet (AA0). A similar behavior of CG decrease was found by Valente et al. [84] when they included NTs as an additive in diets for European seabass (*Dicentrarchus labrax*). The GC, present along the entire intestine, are responsible for the synthesis and secretion of the protective mucus layer that covers the epithelium surface. This mucus layer acts as a medium for protection, lubrication, and transport between the luminal contents and the epithelial lining, and it is an integral structural component of the intestine [85,86].

The capability of fish to digest and use nutrients depends on some factors: digestive enzymes [87], the size of the fish, as well as the length of the intestine. The diet composition and the raw ingredients used in it can modulate the intestinal enzymatic profile [53], while the activity of these enzymes in the digestive tract can be utilized as an indicator of digestive capacity (the ability of fish intestine to take advantage of different nutrients) and the nutritional status of the fish [88]. In this study, the digestive enzymatic activities of the proteases presented the best results in fish fed the diets whose percentage of inclusion of additives (NT concentrate and HPM) was lower. It is worth emphasizing again that there were no major differences between the diets with additives (P1, P2, N250, and N500), while the control diets (AA0 and FM100) did present differences between them. Pepsin activity did not show significant differences, although the values of diets P1 and N250 were numerically higher than that of the control diet

(AA0). The results found in trypsin activity coincide with those obtained by Yang et al. [61], in diets with low doses of HPM equivalents for hybrid grouper. It is well documented that feeding stimulants increase the secretion of different digestive enzymes. Morimoto Kofuji et al. [68] reported that supplementation of feeding stimulants (Alanine, proline, and IMP mixtures) increases the pepsin, trypsin, and chymotrypsin secretions in yellowtail (*Seriola quinqueradiata*). In white shrimp (*Litopenaeus vannamei*, [89]) and seabass (*D. labrax*, [90]), ingesting feed with an appropriate number of small peptides can increase digestive enzyme activity in the intestine and liver. Active peptides can directly act as neurotransmitters to indirectly stimulate intestinal hormone receptors or promote enzyme secretion, and can also provide a complete nitrogen framework for rapid synthesis of digestive enzymes in the body [91].

Regarding the activity of the α-amylase enzyme, no significant differences were found, but the results indicate that the control diet (AA0), fishmeal-free diet, was numerically higher than the diets with inclusion of HPM (P1 and P2). Similarly, Yang et al. [92] found that amylase activity was significantly higher in the control group (HPM0) than in groups with 3, 6, and 9% HPM inclusion in the low-fishmeal feed for hybrid groupers (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂). Amylase activities of tissues and intestinal contents vary among species and appear higher in herbivorous and omnivorous fish than in carnivorous fish [93–96]. In a number of fish species, activities of intestinal α-amylase correlate positively with dietary carbohydrate level and feeding intensity [97]. It is known that the plant carbohydrates may be well digested if the cellular membrane is partially broken, exposing the content of cells to the digestive enzymes. In seabream, this initial process of hydrolysis may be ensured by the acid environment of the stomach [98]. Further hydrolysis of starch is completed in the intestine by the action of amylase [99]. In contrast to mammals, where amylase is produced by salivary and pancreatic cells, the only source of α-amylase in fish appears to be the exocrine pancreas [100]. Previous studies have shown that carnivorous marine fish species (redfish, seabream, and turbot) have the ability to digest starch. This activity was present throughout the gut (including the pyloric ceca), so it was possible to establish the ability to hydrolyze carbohydrates (regardless of their feeding habits) in these species [101]. The results obtained by Alarcón et al. [99] point to the existence of a well-developed carbohydrase activity in the gut of seabream, which is confirmed by the presence of carbohydrate activity in the gut (detected

in different gut sections, from the stomach to the rectum, but mainly present in the pyloric caeca and the intestine). In our study, the high α-amylase activity in the AA0 diet could be due to the ability of seabream to hydrolyze carbohydrates.

Concerning ALP, a dominant enzyme of the intestinal BB, the activity in fish fed the diets supplemented with HPM (P2 and P1) was significantly higher than in fish fed the control diet (AA0). The activity of the digestive enzymes constitutes a considerable factor in the digestion and absorption of the food, especially those found in the BB section of the intestine which are responsible for the final stages of degradation and assimilation of the food [102] and their activity is considerably regulated by the intraluminal presence of dietary substrates [103–105]. ALP is found fundamentally in cell membranes where active transport takes place, which is why it is thought of as a general marker of nutrient absorption [106] and as a marker of intestinal integrity [107]. The functional objective of this enzyme is very far from being completely understood. Nevertheless, it hydrolyzes phosphoester bonds in various organic compounds, such as proteins, lipids, and carbohydrates [108]. The high ALP activity in our study could be due to the content of polypeptides, oligopeptides, small peptides, and free AA present in HPM, and this source of animal protein could have increased the activity of this enzyme.

5. Conclusions

The results of this study demonstrate that both dietary supplementation with HPM as well as NT concentrate supplementation in feed for gilthead seabream can improve growth performance. When HPM and NT concentrates are included in low doses (1% and 250 ppm, respectively), an improvement in the nutritive efficiency indices as well as in the digestive enzymatic activities of proteases is observed, especially with P1.

With these results, we hope to generate a contribution to the active search for a wide variety of additives that manage to materialize improvement in the health status and production performance of aquatic animals, for better economic growth of the aquaculture industry given the current increase in costs and low quantity of fish feed.

6. References

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CAPÍTULO 3

Fishmeal substitution by Iberian pig meal and vegetable proteins blend and inclusion of *Isochrysis* aff. *galbana* (T-Iso) in diets for gilthead seabream (*Sparus aurata* L.): Effects on growth and feed utilization efficiency

Fishmeal substitution by Iberian pig meal and vegetable proteins blend and inclusion of *Isochrysis* aff. *galbana* (T-Iso) in diets for gilthead seabream (*Sparus aurata* L.): Effects on growth and feed utilization efficiency

Glenda Vélez-Calabria, Silvia Martínez-Llorens, María C. Milián-Sorribes, Ignacio Jauralde, Miguel Jover-Cerdá and Ana Tomás-Vidal

Aquaculture and Biodiversity Research Group. Institute of Science and Animal Technology. Universitat Politècnica de València, Valencia, Spain.

Aquaculture Nutrition 2021, *27*, 2169–2181

Abstract

Research was carried out into the effect of partial and total fishmeal (FM) replacement by a vegetable and animal proteins blend as well as the inclusion of microalgae in diets for gilthead seabream (*Sparus aurata* L.). Fish of 64 g on initial weight were fed until apparent satiation for 88 days. The control diet (FM100) contained FM as the main protein source, while in FM25, FM10 and FM0 diets, the FM was replaced 75%, 90% and 100%, respectively, by a proteins blend consisting of Iberian pig meal (IPM) and vegetable protein meals. FM0+ was similar to FM0 diet but included 50 g/kg of *Isochrysis* aff. *galbana* (T-Iso). Results obtained in the final body weight and the specific growth rate indicate that the FM25 and FM100 diets achieved similar performances. An improvement in growth performance and nutrient utilization was observed in the FM0+ diet with respect to the FM0 diet. The highest retention efficiencies of protein, energy and essential amino acids were found in FM100 and FM25 diets. In conclusion, up to 75% FM substitution by a vegetable and animal proteins blend in on-growing gilthead seabream is feasible, in addition, the inclusion of *Isochrysis* aff. *galbana* (T-Iso) improves the growth and retention efficiencies in a non-FM diet.

Keywords: growth performance, nutrition, plant protein, microalgae, aquafeeds, antinutritional factors

1. Introduction

Aquaculture of gilthead seabream (*Sparus aurata* L.) is carried out in 20 countries and it is the principal species in production in the Mediterranean Sea. The aquaculture seabream harvest in Spain in 2019 was 13.521 t and the total aquaculture production in Europe and the rest of the Mediterranean reached 252.406 t (APROMAR, 2020), positioning it as a species of great economic importance for the aquaculture industry.

With the rapid intensification of aquaculture production in the world, the demand for aquafeeds and their main protein ingredient, fishmeal (FM), is increasing exponentially, given that this raw material still remain the principal sources of high-quality protein utilized in feed for carnivorous fish. This continuous increase in demand, together with the decrease in the supply of FM, has led the aquaculture sector to the need to find new alternatives for partial or total FM replacement in fish diets, which should be economic, environmentally friendly, safe, sustainable, and palatable for fish species (Shafique et al., 2021). Consequently, the aquaculture industry and academia have been focused on the search for alternative raw ingredients, in order to reduce the dependency on this ingredient, seeking to become as economically sustainable as possible. Currently, plant-based proteins together with processed animal proteins (PAPs) from non-ruminant animals (poultry and pigs) are used as ingredients in formulated fish feeds, to meet the fish's nutritional requirements for their good digestibility and palatability, lower carbon footprint and reduced levels of antinutritional factors (ANFs) than vegetable products, which improves fish health and welfare (Lanes et al., 2021).

Studies with high replacement of FM by mixture of plant-proteins or plant and animal proteins have produced good results in growth performance and feed utilization, but other important parameters such as survival (Estruch et al., 2018) and quality have been affected. In the case of survival, the impact is generally attributed to the presence of ANFs present in plant sources (Francis et al., 2001), hence the current study of new alternatives, try to minimize this effect by the use of a mix of animal and plant protein (PP), as well as food additives.

Studies carried out with animal protein sources in diets for cultured marine fish are scarce. Animal by-products are potential alternative ingredients for FM and are largely available, such as meat and bone meal (MBM), poultry by-product meal (PBM), feather meal, and

blood meal. A provisional solution to reduce production costs lies in the identification of low-price food items, easily affordable and with no interest for human markets. The quality of the proteins in the meals from animal by-products will vary according to the origin of the raw materials; meat protein would have better quality than other tissues such as tendon or skin; therefore, it is necessary to measure protein quality in animal by-products meals. In addition, animal by-product meal contains a reasonable amount of phosphorus, an important nutrient for aquatic animals (Tangendjaja, 2015).

The use of PAP in aquafeeds is widely varied depending on the region in which they are utilized. In the European Union (EU), its use was prohibited from 1990–2000 (Regulation (EC) No 999/2001 of the European Parliament and of the Council of 22 May 2001), due to the appearance of bovine spongiform encephalopathy in ruminants in the 1980s-1990s. Despite this, in 2013 this restriction was partially lifted authorizing the use of PAP derived from non-ruminant animals for feeding aquaculture animals (Commission Regulation (EU) No 56/2013 of 16 January 2013). This allowed access to a new range of ingredients that can be widely used in aquafeeds (Moutinho et al., 2017a). The quality of these terrestrial animal protein sources is highly dependent on the quality of the raw material as well as the processing to which it has been exposed. Use of more suitable processing technologies, in particular drying techniques, has made it possible to produce more specific and selected products for the formulation of fish diets (Bureau et al., 2000; Bureau et al., 1999). For example, co-extrusion and flash drying are currently used to produce superior quality meat and bone, and poultry by-products (Hernández et al., 2008). However, the technological process for the production of PAP was reviewed (EC No. 94/449; pressure, 3 bar by steam for 20 min; maximum particle size, 50 mm; temperature higher than 133°C), which could lead to compromising their nutritional quality. Accordingly, it is necessary to thoroughly assess these new ingredients (Moutinho et al., 2017a).

The Iberian pig is considered to be the most valuable Mediterranean breed of pig due to its considerable population size as well as its economic importance (Álvarez et al., 2014; Juárez et al., 2009). Pig meat production in Spain amounted to more than 52.9 million slaughtered animals and about 4.64 million tons of meat produced in 2019, figures that keep Spain in the fourth position in the world. The Iberian pig census represented 10.8% (3.3 million animals in December 2019) of the total pig census in Spain (31.2 million

animals in December 2019). Since 2015, production in Spain has grown by 20%, giving an idea of the huge growth that the pig sector is experiencing at the national level (MAPA, 2020). According to the above, it could be estimated that the large volumes of slaughterhouse waste would allow a constant availability of Iberian pig by-products for the production of Iberian pig meal for aquafeeds, which would help reduce the production costs as well as ensuring sustainability of the sector. The Iberian pig is an autochthonous variety from the Iberian Peninsula pig whose particularity is based on its high quality of fat and flavor (Lopez-Bote, 1998), as well as in its high rusticity (hereditary resistance to non-optimal conditions of the environment) (Martinez-Macipe et al., 2016). Furthermore, the Iberian pig carcass is highly prized in the market, based on an outstanding balance of fatty acids in its lipid deposits - intramuscular and subcutaneous fat - especially, subcutaneous fat. Indeed, in the Iberian pig sector, a lower proportion of palmitic acids (C16:0), stearic (C18:0) and linoleic (C18:2 n-6) and a high proportion of oleic acid (C18:1 n-9) in the carcasses are utilized as quality indicators (De Pedro, 2001; Tejerina et al., 2012). Iberian pig by-product meal could position itself as an emerging ingredient for aquaculture feeds in Spain, due to its nutritional characteristics, availability and ease of use.

A wide variety of additives are used in aquaculture that have great beneficial effects on the host, such as fighting disease, improving growth and, in some cases, acting as alternative antimicrobial compounds (Irianto and Austin, 2002), as well as stimulating the immune response of the host. Moreover, the amount of research into the development of new strategies in food supplementation has increased, which can be evaluated in the introduction of various compounds that promote health and growth, such as probiotics, prebiotics, symbiotics, phytobiotics and other functional food supplements (Akhter et al., 2015; Denev, 2008).

Microalgae comprise an extensive group of photosynthetic heterotrophic organisms, many of which are rich in protein, lipids, and bioactive compounds (Yarnold et al., 2019), which are classified according to certain characteristics, such as cell structure, pigments, and substances (Cerezuela et al., 2012). Depending on the algal species and their growth conditions, they can contain up to 60% protein, 60% carbohydrates, or 70% oils (Draaisma et al., 2013) and produce valuable pigments, growth-promoting substances,

and hormones as well as secondary metabolites that provide natural antioxidant, antimicrobial, anti-inflammatory, and immunostimulant benefits to aquatic animals (García-Chavarría and Lara-Flores, 2013; Michalak and Chojnacka, 2015). In addition, they have the ability to synthesize all amino acids (thus providing those which are essential to animals and humans); existence of carbohydrates in the form of starch, cellulose, sugars, and other polysaccharides; lipids in the form of fatty acids of the n3 and n6 families and glycerol; and an important content of many essential vitamins (A, B1, B2, B6, B12, C, E, biotin, pantothenic acid and folic acid), minerals (iron, selenium, zinc, magnesium, calcium, phosphorus) and antioxidant substances (Borowitzka, 1997; Cerezuela et al., 2012; Duerr et al., 1998). Currently, microalgae may play important roles in feed (for cattle, poultry, shellfish, and fish), food additives, FM and oil replacement, coloring of salmonids, inducers of biological activities, and enhancers of nutritional value of zooplankton fed to fish larvae and fry (Camacho et al., 2019; Dineshbabu et al., 2019; Guedes et al., 2015; Valente et al., 2021; Yarnold et al., 2019). All these particularities have led to further exploration of new functional ingredients from microalgae with the purpose of providing an additional health benefit in addition to the energy and nutritional aspects of food (Christaki et al., 2011; Plaza et al., 2009; Spolaore et al., 2006). The microalgae-derived materials are made up of bioactive compounds. Their bioactivity can be selected from one or more of immune-enhancement, growth promotion, disease resistance, antiviral and antibacterial action, improved gut function, probiotic colonization stimulation, as well as enhanced feed conversion, reproductive performance and weight control (Harel et al., 2007; Madeira et al., 2017; Yarnold et al., 2019). The reports of anti-inflammatory effects on rats due to *I. galbana* (Nuño et al., 2013) may correspond to the action of bioactive compounds in *I. galbana*, including eicosapentanoic acid (EPA) and other than EPA (Bonfanti et al., 2018). These bioactive compounds may be protein, polyunsaturated fatty acids, carotenoids, vitamins and minerals (Camacho et al., 2019). The content of vitamin C (ascorbic acid), present in *Isochrysis* aff. *galbana* (T-Iso) as a bioactive compound, amounts to 885 mg per kg DW (Bandarra et al., 2003). The properties of this bioactive compound benefit gastrointestinal physiology and lipid metabolics (Nuño et al., 2013), hypocholesterolemic potential (Dvir et al., 2009) and antioxidant action (Matos et al., 2017). Likewise, other studies confirm that *I. galbana* result highly digestible and its nutrients support the growth of gilthead seabream

(Palmegiano et al., 2009) and its inclusion in the diets for European sea bass does not adversely affect feed intake and growth performance (Tibaldi et al., 2015).

For these reasons, the aim of this present work was to evaluate the effect of FM substitution by a vegetable and animal proteins blend, as well as the inclusion of the microalgae *I.* aff. *galbana* (T-Iso) on the growth performance, feed utilization efficiency and protein efficiency (protein and amino acids retention) of gilthead seabream (*S. aurata*).

2. Materials and methods

The experimental protocol implemented in this trial was reviewed and approved by the Committee of Ethics and Animal Welfare of the Universitat Politècnica de València (code: P4-04-05-2017). All experiments were carried out in an accredited animal care facility (code: ES462500001091) in accordance with the Spanish Animal Protection Regulations RD 53/2013, which complies with European Union Directive 2010/63 with regard to the protection of animals used for experimental and other scientific purposes.

2.1. Experimental diets

Four isonitrogenous (450 g/kg crude protein) and isolipidic (200 g/kg crude lipid) experimental diets were formulated with different levels of FM replacement and were named as FM25, FM10, FM0 and FM0+. In addition, a control diet (FM100), whose ingredients were FM (as the protein source), wheat, fish and soy oils and a complex of vitamins and minerals was used. In the FM25, FM10, FM0 and FM0+ diets, FM was replaced at a proportion of 75%, 90% and 100%, respectively, by an animal and vegetable proteins blend consisting in Iberian pig meal (IPM), pea, sunflower, and soybean meal. Additionally, microalgae *I.* aff. *galbana* (T-Iso), provided by Marine Microalgae Biotechnology Research Group of the University of Almeria (Spain), was included at 50 g/kg in the FM0+ diet. To cover the essential amino acids (EAA) needs, methionine (Met) was added using the reference of amino acids (AA) requirements of *S. aurata* reported by Peres and Oliva-Teles (2009). Ingredients and chemical composition of the experimental diets are presented in Table 1.

Before formulating the diets, a chemical analysis of each of the ingredients was carried out, they were weighed individually and then mixed to homogenize the mixture.

Subsequently, the diets were prepared using a cooking-extrusion process with a semi-industrial twin screw extruder (CLEXTRAL BC-45, St. Etienne, France) at the UPV facilities.

Table 1. Formulation and proximate composition of the experimental diets

	Experimental diets				
	FM100	**FM25**	**FM10**	**FM0**	**FM0+**
Ingredients (g/kg)					
Fishmeal [a]	590	150	60		
Wheat meal [b]	259	56	14		
Soybean meal [c]		171	206	220	206
Pea meal [d]		101	122	129	111
Sunflower meal [e]		101	122	129	111
Iberian pig meal [f]		237	288	328	328
Microalgae *I.* aff. *galbana* (T-Iso) [g]					50
Soybean oil	96	56	50	41	41
Fish oil	45	85	90	100	100
Mono calcium phosphate		28	33	38	38
L-Methionine [h]		5	5	5	5
Multivitamin and minerals mix [i]	10	10	10	10	10
Ratio FM:PP:IPM	0.94:0.06:0	0.24:0.33:0.43	0.1:0.38:0.53	0:0.4:0.6	0:0.38:0.62
Analyzed composition (g/kg dry weight)					
Dry matter (DM)	908.6	916.6	905.0	908.8	902.6
Crude Protein (CP)	472.0	465.1	471.4	470.4	459.8
Crude Lipid (CL)	198.9	190.6	185.6	186.7	195.3
Ash	111.1	83.6	76.6	89.1	89.1
Calculated values					
Crude Fiber (CF, g/kg) [j]	8.0	33.0	38.1	39.9	34.9
Energy (kJ/g) [k]	21.78	23.34	23.26	23.68	23.45
NFE (g/kg) [l]	210.0	227.7	228.3	213.9	220.9

[a] Fishmeal (g/kg): (932 DM, 707 CP, 89 CL, 151 Ash); Vicens I Batllori S.L., Girona, Spain.

[b] Wheat meal (g/kg): (890 DM, 116 CP, 15 CL, 18 Ash); DESCO, Museros, Valencia, Spain.

[c] Soybean meal (g/kg): (882 DM, 499 CP, 22 CL, 71 Ash); DESCO, Museros, Valencia, Spain.

[d] Pea meal (g/kg): (866 DM, 216 CP, 10 CL, 39 Ash); DESCO, Museros, Valencia, Spain.

[e] Sunflower meal (g/kg): (896 DM, 291 CP, 15 CL, 67 Ash); DESCO, Museros, Valencia, Spain.

[f] Iberian pig meal (g/kg): (959 DM, 804 CP, 163 CL, 19 Ash); Slaughterhouse Guijuelo S.A. – Maguisa, Salamanca, Spain.

[g] Microalgae *I.* aff. *galbana* (T-Iso) (g/kg): (889.8 DM, 350 CP, 10.9 CL, 29.7 Ash); Biotechnology research group of the University of Almeria, Spain.

[h] L-Methionine: Guinama®.

[i] Multivitamin and minerals mix (values are g/kg): Premix: 25; Choline, 10; DL-α-tocopherol, 5; ascorbic acid, 5; $(PO_4)_2Ca_3$, 5. Premix composition: retinol acetate, 1000000 IU/kg; calciferol, 500 IU/kg; DL-α-tocopherol, 10; menadione sodium bisulfite, 0.8; thiamine hydrochloride, 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.

[j] Crude Fiber, CF (g/kg) was calculated by FEDNA (2010) tables.

[k] Energy (kJ/g) = (51.8 x (%C/100)) – (19.4 x (%N/100)). Calculated according to Brouwer (1965).

[l] NFE, Nitrogen-free extract (g/kg) = 100 − CP − CL − CF − Ash.

The processing conditions were as follows: a pressure of 4-5 Mpa, a temperature of 110°C and a screw speed of 100 rpm. All feed ingredients and the experimental diets were analyzed in triplicate.

2.2. Growth trial and fish sampling

Gilthead seabream (*S. aurata*) juveniles were provided by a local fish farm (Alevines del Mediterráneo, S. L. (Blaumar), Sagunto, Spain) and transported to the Fish Nutrition Laboratory of the UPV, Spain. Before starting the feeding test, all fish were acclimated to indoor rearing conditions for four weeks and fed a standard diet for seabream (480 g/kg crude protein, CP; 230 g/kg crude lipid, CL; 110 g/kg ash; 22 g/kg crude fiber, CF; and 140 g/kg nitrogen free-extract, NFE). After the acclimation period, gilthead seabream juveniles (initial average weight: 64 ± 1.3 g, mean ± standard error of the mean) were redistributed in 15 cylindrical fiberglass tanks (three per treatment) in groups of 24 fish per tank. The capacity of each tank was 1750 L.

The duration of the experiment was 88 days. The experiment was carried out in a seawater recirculation system (65 m^3 capacity) that had a rotary mechanical filter and a gravity biofilter (approximately 6 m^3). The water temperature was kept at 21 ± 0.82°C, dissolved oxygen was 7.1 ± 0.73 mg L^{-1}, salinity was 33 ± 2.15 g L^{-1}, and pH fluctuated between 8.0 to 8.5 during the experiment. All tanks had aeration supply. The water temperature was kept constant with the help of a heat/cold specific pump installed in the system. The photoperiod was natural and all tanks maintained similar lighting conditions.

Fish were observed daily and were weighed at 28-day intervals to determine growth parameters. Before weighing, all fish were, fasted for 41 hours and anesthetized with 30 mg L^{-1} of clove oil (Guinama®, Valencia, Spain) that contain 87% of eugenol. Fish were fed by hand twice a day (09:00 and 16:00 hours) until apparent satiation from Monday to Saturday, with fasting on Sunday. Pellets were distributed slowly, allowing all fish to eat. Feed intake (FI) was recorded daily. The uneaten diet was collected and dried to determine FI.

At the end of the feeding trial, all the fish were individually weighted. Five fish from each tank, as well as five fish from the initial stock, were randomly slaughtered using a lethal bath of clove oil (150 mg L^{-1}), for the determination of biometric parameters and whole-

body proximate composition. The samples from each tank were pooled and stored at -30°C. Fish total weight and length, as well as viscera, visceral fat and liver weights were recorded for determination of condition factor (CF), viscerosomatic (VSI), visceral fat (VFI), and hepatosomatic (HSI) indexes.

The growth performance indicators and retention efficiencies of ingested protein (PIR), energy (EIR) and essential amino acids (AAIRE) were determined at the end of the experiment and the tank was used as an experimental unit. The specific growth rate (SGR), FI, feed conversion ratio (FCR) and protein efficiency ratio (PER) were obtained taking into account the monthly reported biomass of dead fish. The biometric parameters were obtained at the end of the growth trial, using five fish per tank, 15 per treatment.

2.3. Chemical analyses

Fish diets, feed ingredients, and proximate composition of whole fish were analyzed in accordance with the AOAC (2002) procedures: dry matter, official method 934.01 (105°C to constant weight); crude protein, official method 990.03 (analyzed by direct combustion method DUMAS using LECO CN628); crude lipid, official method 920.39 (extracted with methyl-ether using ANKOM^{XT10} Extractor) and ash, official method 942.05 (incinerated at 550°C for 5 h). All analyses were performed in triplicate.

2.4. Amino acids analyses

Based on the method described by Bosch et al. (2006), the AA contents of the ingredients, diets and fish carcasses, were determined using a Waters HPLC system (Waters 474, Waters, Milford, MA, USA) consisting of an auto sampler (Model 717, Waters), a fluorescence detector (Model 474, Waters), two pumps (Model 515, Waters), and a temperature control module. Aminobutyric acid was added as an internal standard prior to hydrolyzation. Amino acids were derivatised with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Cysteine and Met were determined separately as cysteic acid and methionine sulphone following oxidation with performic acid. Amino acids were separated with a C-18 reverse-phase column Waters Acc. Tag (150 mm × 3.9 mm) and then converted to Cys and Met. The AA composition of the diets and main protein sources used can be seen in Table 2.

2.5. Statistical analyses

Prior to analysis, all variables were checked for normal distribution with the Kolmogorov–Smirnov test and homogeneity of variances by the Levene test. Growth data, nutrient utilization, biometric parameters, body composition and amino acid composition and retention were treated using multifactor analysis of variance (ANOVA). Student Newman–Keuls test was used to assess specific differences among diets. Data were considered statistically significant when $p < .05$ and the data is shown as the mean ± pooled standard error of the mean (SEM). Mean values for each tank were the units of observation for statistical evaluation (five fish per tank, three tanks per treatment). Statistical data analyses were performed using Statgraphics, Statistical Graphics System, Version Centurion XVI, Warrenton, Virginia, USA.

Table 2. Amino acids composition of the experimental ingredients and diets

	Ingredients		**Experimental diets**				
	FM	**IPM**	**FM100**	**FM25**	**FM10**	**FM0**	**FM0+**
EAA (g/kg dry weight)							
Arginine	56.0	51.1	30.4	30.4	29.1	27.8	29.0
Histidine	24.2	11.5	9.10	6.90	6.30	6.90	6.80
Isoleucine	32.5	24.1	18.6	15.8	15.4	15.0	15.4
Leucine	62.6	46.5	31.3	27.7	27.0	26.4	27.2
Lysine	57.4	43.0	28.1	20.7	21.2	20.8	19.2
Methionine	22.0	9.80	11.1	11.5	9.30	9.20	10.0
Phenylalanine	35.6	26.0	16.1	15.3	14.5	14.6	15.9
Threonine	33.9	17.6	16.1	12.8	12.4	11.5	12.7
Valine	37.0	38.2	21.8	19.8	19.7	19.7	19.6
NEAA (g/kg dry weight)							
Alanine	41.3	64.4	22.9	24.3	25.9	25.4	24.8
Aspartate	66.5	65.5	32.7	36.9	39.0	38.7	35.9
Cysteine	5.3	2.3	5.1	4.1	3.2	3.6	3.7
Glutamate	95.5	119.2	56.8	60.5	64.8	62.2	59.1
Glycine	40.7	149.1	24.8	43.8	46.0	46.6	48.1
Proline	27.4	87.6	17.8	29.0	31.3	31.3	33.3
Serine	32.6	25.5	16.1	15.3	14.7	15.1	15.4
Tyrosine	25.5	16.8	11.7	9.40	9.60	8.70	9.40
EAA	361.2	267.7	182.6	160.9	154.9	151.9	155.9
NEAA	334.8	530.4	188.0	223.4	234.6	231.5	229.7
EAA/NEAA	1.08	0.50	0.97	0.72	0.66	0.66	0.68

FM, fishmeal; IPM, Iberian pig meal; EAA, essential amino acids; NEAA, non-essential amino acids.

3. Results

3.1. Fish growth and feed utilization efficiency

The results obtained on growth performance and feed utilization efficiency are shown in Table 3. At the end of the growth period, gilthead seabream fed with the non-fishmeal diet (FM0) presented by some margin the lowest final body weight, WG and SGR (137.4 g, 110.6% and 0.8% day^{-1}, respectively), whereas fish fed the FM25 diet and fish fed the control diet (FM100) showed the highest growth (SGR, 1.2 and 1.4% day^{-1}, respectively). Fish fed the FM0+ and FM10 diets attained a higher weight and SGR than those fed the FM0 diet. All diets were well accepted and no significant differences between groups for FI were detected. The FCR was higher (2.1) in fish fed the FM0 diet, and the FM0+ diet did not show a difference with the FM100, FM25 or FM10 diet (1.6 and 1.5, 1.5, 1.7 respectively). Significant differences were found in PER, being the lowest in fish fed the FM0 diet, while the FM0+ diet showed no difference with the FM100 and FM25 diets.

Table 3. Growth performance, nutrient utilization and biometric parameters of gilthead seabream fed with different experimental diets

	Experimental diets				
	FM100	**FM25**	**FM10**	**FM0**	**FM0+**
Initial weight (g)	63.1 ± 1.84	64.1 ± 1.84	64.1 ± 1.84	65.4± 1.84	63.4 ± 1.84
Final weight (g)	214.7 ± 12.39^{a}	193.2 ± 12.39^{a}	162.9 ± 12.39^{ab}	137.4 ± 12.39^{b}	175.3 ± 12.39^{ab}
WG (%) [a]	239.5 ± 14.54^{a}	200.1 ± 14.54^{ab}	153.7 ± 14.54^{bc}	110.6 ± 14.54^{c}	176.7 ± 14.54^{b}
SGR (% day^{-1}) [b]	1.4 ± 0.06^{a}	1.2 ± 0.06^{ab}	1.1 ± 0.06^{b}	0.8 ± 0.06^{c}	1.2 ± 0.06^{b}
FI (g 100 g fish^{-1} day^{-1}) [c]	1.8 ± 0.07	1.6 ± 0.07	1.7 ± 0.07	1.7 ± 0.07	1.7 ± 0.07
FCR [d]	1.5 ± 0.12^{b}	1.5 ± 0.12^{b}	1.7 ± 0.12^{ab}	2.1 ± 0.12^{a}	1.6 ± 0.12^{b}
PER [e]	1.6 ± 0.10^{a}	1.6 ± 0.10^{a}	1.4 ± 0.10^{ab}	1.1 ± 0.10^{b}	1.5 ± 0.10^{a}
CF (g cm^{-3}) [f]	2.1 ± 0.05^{a}	1.9 ± 0.05^{bc}	1.8 ± 0.05^{bc}	1.7 ± 0.05^{c}	1.9 ± 0.05^{b}
VSI (%) [g]	7.7 ± 0.27	7.9 ± 0.27	8.0 ± 0.27	8.3 ± 0.27	8.8 ± 0.27
HSI (%) [h]	1.3 ± 0.07	1.0 ± 0.07	1.0 ± 0.07	1.0 ± 0.07	1.0 ± 0.07
VFI (%) [i]	1.8 ± 0.20	1.4 ± 0.20	1.4 ± 0.20	2.1 ± 0.20	1.9 ± 0.20

[a] Weight gain (WG, %) = 100 × (final weight - initial weight) / initial weight.

[b] Specific growth rate (SGR, % day^{-1}) = 100 × ln (final weight / initial weight)/days.

[c] Feed intake (FI, g 100 g fish^{-1} day^{-1}) = 100 × feed consumption (g) / average biomass (g) × days.

[d] Feed conversion ratio (FCR) = feed offered (g) / weight gain (g).

[e] Protein efficiency ratio (PER) = weight gain (g) / protein offered (g).

[f] Condition factor (CF, g cm^{-3}) = 100 × total weight (g) / total length^{3} (cm).

[g] Viscerosomatic index (VSI, %) = 100 × visceral weight (g) / fish weight (g).

[h] Hepatosomatic index (HIS, %) = 100 × liver weight (g) / fish weight (g).

[i] Visceral fat index (VFI, %) = 100 × visceral fat weight (g) / fish weight (g).

Data are presented as mean ± *SEM* (n = 3, growth performance and nutrient utilization; n = 15, biometric parameters). Different superscript letters indicate significant differences among treatments ($p < .05$).

3.2. Biometric indexes and body composition

Regarding biometric parameters (Table 3), statistical differences were detected in condition factor (CF), fish fed the FM0+ diet obtained a higher value (1.9 g cm^{-3}) than fish fed the FM0 diet (1.7 g cm^{-3}), similar to those fed the FM10 and FM25 diets and however lower than the FM100 (2.1 g cm^{-3}). No differences were observed in the viscerosomatic index (VSI), hepatosomatic index (HSI), and visceral fat index (VFI).

The proximate composition of the whole-body, expressed as g/kg of the wet weight, is shown in Table 4. Fish fed the FM100 and FM0 diets exhibited the lowest moisture content (664.8 and 674.1 g/kg, respectively), and accordingly, the lipid content of those fish were the highest (127.1 and 130.2 g/kg, respectively). No significant differences for whole-body protein and ash contents were found.

Table 4. Proximate composition of whole body gilthead seabream fed with different experimental diets

	Initial	Experimental diets				
		FM100	FM25	FM10	FM0	FM0+
Analyzed composition (g/kg wet weight)						
Moisture	665.0	664.8 ± 0.32^{c}	687.7 ± 0.32^{a}	680.5 ± 0.32^{ab}	674.1 ± 0.32^{bc}	688.1 ± 0.32^{a}
Crude Protein (CP)	169.0	175.0 ± 0.28	170.4 ± 0.28	171.4 ± 0.28	168.9 ± 0.28	167.9 ± 0.28
Crude Lipid (CL)	123.8	127.1 ± 0.28^{a}	110.2 ± 0.28^{b}	117.7 ± 0.28^{b}	130.2 ± 0.28^{a}	112.9 ± 0.28^{b}
Ash	33.0	31.7 ± 0.14	30.4 ± 0.14	28.4 ± 0.14	27.9 ± 0.14	29.2 ± 0.14

Data are presented as mean ± *SEM* ($n = 3$). Different superscript letters indicate significant differences among treatments ($p < .05$).

3.3. Amino acids composition and retention efficiencies

No significant differences were observed in the whole-body AA content of the fish as shown in Table 5, except for the non-essential amino acid aspartate which had the highest value in fish fed the FM100 diet (14.1 g/kg) and the lowest value in fish fed the FM0 diet (12.2g/kg).

Table 5. Amino acids composition of whole-body of gilthead seabream after feeding different experimental diets

		Experimental diets				
	Initial	FM100	FM25	FM10	FM0	FM0+
EAA (g/kg wet weight)						
Arginine	14.5	12.9 ± 0.04	12.1 ± 0.15	11.7 ± 0.08	11.8 ± 0.06	11.7 ± 0.09
Histidine	3.30	2.80 ± 0.01	2.80 ± 0.07	3.30 ± 0.14	2.50 ± 0.04	2.60 ± 0.02
Isoleucine	4.90	6.80 ± 0.02	6.70 ± 0.09	6.50 ± 0.04	6.20 ± 0.04	6.20 ± 0.02
Leucine	12.9	11.7 ± 0.04	11.6 ± 0.12	11.5 ± 0.07	11.0 ± 0.04	11.1 ± 0.03
Lysine	12.7	10.8 ± 0.16	10.5 ± 0.05	10.4 ± 0.15	9.20 ± 0.02	9.90 ± 0.06

Methionine	4.30	3.90 ± 0.005	3.80 ± 0.02	3.60 ± 0.03	3.70 ± 0.03	3.70 ± 0.02
Phenylalanine	5.40	6.00 ± 0.07	5.90 ± 0.12	5.70 ± 0.04	5.80 ± 0.08	5.90 ± 0.10
Threonine	7.00	6.20 ± 0.01	6.30 ± 0.11	6.30 ± 0.03	5.90 ± 0.03	6.10 ± 0.03
Valine	7.10	8.10 ± 0.02	7.90 ± 0.09	7.70 ± 0.04	7.40 ± 0.03	7.30 ± 0.01
NEAA (g/kg wet weight)						
Alanine	13.1	8.70 ± 0.07	8.80 ± 0.07	8.60 ± 0.05	8.40 ± 0.05	8.50 ± 0.01
Aspartate	18.4	14.1 ± 0.11^{a}	13.4 ± 0.13^{ab}	13.5 ± 0.12^{ab}	12.2 ± 0.04^{b}	12.7 ± 0.09^{ab}
Cysteine	1.40	1.40 ± 0.01	1.30 ± 0.02	1.10 ± 0.01	1.30 ± 0.02	1.30 ± 0.01
Glutamate	27.2	20.4 ± 0.14	20.2 ± 0.17	20.4 ± 0.13	18.9 ± 0.04	19.7 ± 0.13
Glycine	15.5	11.0 ± 0.04	11.6 ± 0.09	10.7 ± 0.04	11.5 ± 0.01	11.3 ± 0.20
Proline	8.90	6.20 ± 0.04	6.20 ± 0.02	5.80 ± 0.01	6.30 ± 0.02	6.00 ± 0.05
Serine	8.10	6.10 ± 0.02	6.70 ± 0.12	6.00 ± 0.01	6.00 ± 0.04	5.90 ± 0.03
Tyrosine	5.10	5.30 ± 0.08	4.80 ± 0.09	4.90 ± 0.005	4.70 ± 0.05	4.80 ± 0.06
EAA/NEAA	0.74	0.95 ± 0.08	0.93 ± 0.005	0.94 ± 0.06	0.92 ± 0.05	0.92 ± 0.008

EAA, Essential amino acids; NEAA, Non-essential amino acids. Data are presented as mean ± *SEM* (n = 3). Different superscripts letters indicate significant differences among treatments ($p < .05$).

The retention efficiency of protein (PIR) and energy intake (EIR) were lowest (19.0 and 48.9%, respectively) in fish fed the FM0 diet (Table 6); whereas fish fed the FM100 diet presented the highest efficiencies (28.7 and 76.3%, respectively). In fish fed the FM10 and FM0+ diets, the PIR values did not show differences (23.8 and 25.8%, respectively) as was the case with the EIR values (59.4 and 61.5% respectively).

Table 6. Retention efficiencies of ingested protein, energy and essential amino acids of gilthead seabream fed with different experimental diets

	Experimental diets				
	FM100	**FM25**	**FM10**	**FM0**	**FM0+**
PIR (%) [a]	28.7 ± 2.00^{a}	28.0 ± 2.00^{a}	23.8 ± 2.00^{ab}	19.0 ± 2.00^{b}	25.8 ± 2.00^{ab}
EIR (%) [b]	76.3 ± 5.34^{a}	68.1 ± 5.34^{ab}	59.4 ± 5.34^{ab}	48.9 ± 5.34^{b}	61.5 ± 5.34^{ab}
AAIRE (%) [c]					
Arginine	30.8 ± 2.26^{a}	26.8 ± 2.26^{ab}	22.3 ± 2.26^{ab}	18.0 ± 2.26^{b}	24.8 ± 2.26^{ab}
Histidine	21.8 ± 8.71	26.7 ± 8.71	35.2 ± 8.71	14.1 ± 8.71	22.9 ± 8.71
Isoleucine	31.4 ± 2.27	36.0 ± 2.27	32.1 ± 2.27	26.3 ± 2.27	32.3 ± 2.27
Leucine	27.3 ± 2.23^{ab}	29.6 ± 2.23^{a}	25.8 ± 2.23^{ab}	18.8 ± 2.23^{b}	26.1 ± 2.23^{ab}
Lysine	25.1 ± 3.88^{ab}	31.2 ± 3.88^{a}	23.1 ± 3.88^{ab}	10.8 ± 3.88^{b}	26.6 ± 3.88^{ab}
Methionine	26.1 ± 2.52	23.2 ± 2.52	22.6 ± 2.52	18.6 ± 2.52	23.9 ± 2.52
Phenylalanine	29.7 ± 3.24	30.3 ± 3.24	26.9 ± 3.24	22.6 ± 3.24	27.3 ± 3.24
Threonine	28.0 ± 2.74	35.1 ± 2.74	30.8 ± 2.74	23.2 ± 2.74	31.3 ± 2.74
Valine	30.1 ± 1.85^{a}	31.5 ± 1.85^{a}	26.7 ± 1.85^{ab}	21.1 ± 1.85^{b}	27.2 ± 1.85^{ab}

[a] Retention efficiency of protein intake (PIR, %) = 100 × protein fish gain (g)/protein intake (g).
[b] Retention efficiency of energy intake (EIR, %) = 100 × energy fish gain (g)/energy intake (g).
[c] Retention efficiency of amino acid (AAIRE, %) = 100 × AA fish gain (g)/AA ingested (g).
Data are presented as mean ± *SEM* (n = 3). Different superscript letters indicate significant differences among treatments ($p < .05$).

There were significant differences in EAA retention efficiency for arginine (Arg), leucine (Leu), lysine (Lys) and valine (Val) in gilthead seabream fed the different experimental

diets (Table 5). Fish fed the FM25 diet showed the highest retention for Leu, Lys and Val, and fish fed the FM100 diet showed the highest retention for Arg and Val. The retention efficiency of Lys and Leu did not show differences in the FM100, FM10 and FM0+ diets, as was the case with Arg in the FM25, FM10 and FM0+ diets, and Val in the FM10 and FM0+ diets. Fish fed the FM0 diet presented the lowest values of EAA retention efficiency.

Significant differences were observed in the ratio between the ingested EEA of the experimental diets and the EAA of whole fish, except for EAA phenylalanine (Phe) where no significant differences were observed (Figure 1). Fish fed the FM100 diet showed the highest values in almost all EAA. Arg and Met showed similar values in fish fed the FM100 and FM25 diets. Values of isoleucine (Ile), Leu, Lys, threonine (Thr) and Val followed the same trend for fish fed the FM25, FM10, FM0 and FM0+ diets. Except for Lys in the group fed the FM0+ diet and Thr in the group fed the FM0 diet, the ratio % EAA_{diet}/% EAA_{fish} were all higher than 0.7.

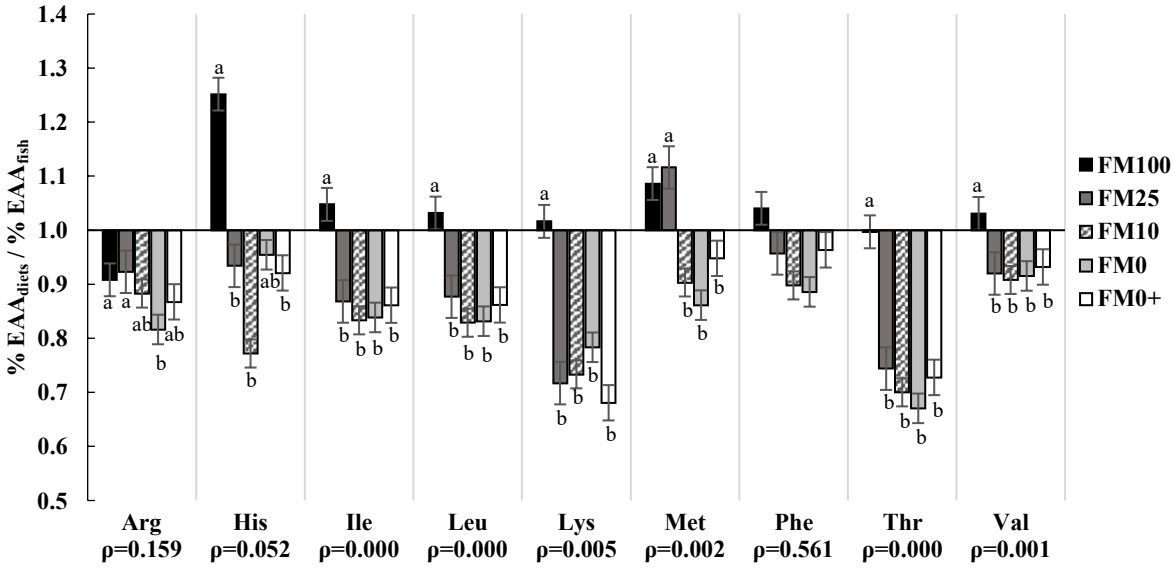

Figure 1. Ratio between ingested EAA of the experimental diets and EAA of whole fish. Each value is the mean of triplicate groups. Significant differences are indicated by different letters ($p < .05$).

4. Discussion

One of the main purposes of replacing FM is to improve sustainability of carnivorous fish production, as well as provide more economical alternatives compared with the high cost of FM. The results of the present study indicate that up to 75% of FM (FM25) can be replaced by an animal and vegetable proteins blend in gilthead seabream (*S. aurata*) diets without compromising feed utilization and growth performance. In a similar way to these results, other studies corroborate the feasibility of high FM substitutions. Dietary FM can be replaced at least up to 83% with PBM, along with a constant mixture of both animal and plant protein sources, in diets for gilthead seabream juveniles without affecting growth performance and feed utilization (Fontinha et al., 2021). The FE showed considerable enhancement and SGR was unchanged or moderately reduced in gilthead seabream fed a with 75% FM replacement diet by PP sources (Sitjà-Bobadilla et al., 2005). Both the FM diet and the diet with a 75% mixture of PP sources in seabream, had similar weight gain and SGR, while FE and PER were significantly higher in the PP diet (De Francesco et al., 2007). No statistical differences were found in the final weight and the nutritional efficiency indexes in seabream, between the FM diet and the diet with a 75% FM substitution with a mixture of plant meals (Estruch et al., 2018).

The diet with total FM replacement and no addition of *I.* aff. *galbana* T-Iso (FM0) showed the lowest values on growth performance. Total FM replacement is widely shown to cause low growth rates (Lunger et al., 2007), poor feed efficiency (Gómez-Requeni et al., 2004), even immunosuppression (Sitjà-Bobadilla et al., 2005), and mortality (Estruch et al., 2015). Even when amino acid is balanced with the blend of sources, not good results are achieved usually especially in aquafeeds designed for high-level carnivores. This effect is mainly explained cause a "small inclusion of FM in the former diet (8% of dietary protein) must have provided some essential nutrients that aided in keeping the fish alive" (Lunger et al., 2007). Contrary to the above, the fishmeal-free diet and addition of the microalgae *I.* aff. *galbana* T-Iso (FM0+) presented a notable improvement in growth performance in relation to the results obtained the FM0 diet. This clearly indicates that the inclusion of *I.* aff. *galbana* (T-Iso) has a positive effect at the same level of substitution, perhaps due it, marine provenance may provide something that is needed by fish, provided usually by FM. This positive effect of the microalgae addition in the FM0+ diet is a relevant finding. The use of microalgae as an additive in aquaculture has received

a lot of attention due to the positive effect on weight gain, increased triglyceride and protein deposition in muscle, improved resistance to disease, improved taste and consistency of flesh, decreased nitrogen output into the environment, increased omega-3 fatty acid content, physiological activity, starvation tolerance, carcass quality, and increase in the rate of growth of aquatic species due to better digestibility (Becker, 2004; Fleurence et al., 2012). As is known, microalgae contain compounds such as carbohydrates, proteins (from 300 to 550 g/kg DM) (González López et al., 2010), minerals, oil, fats, polyunsaturated fatty acids (40% PUFAs, (Batista et al., 2013)) as well as bioactive compounds such as antioxidants (polyphenols, tocopherols [vitamin E], vitamin C, mycosporine-like amino acids), and pigments, such as carotenoids (carotene xanthophyll), chlorophylls, and phycobilins (phycocyanin, phycoerythrin), which possess antibacterial, antiviral, antifungal, antioxidative, anti-inflammatory, and antitumor properties (Michalak and Chojnacka, 2015). According to the above, the improvement in fish growth observed in the FM0+ diet could be related to these properties. Our results did not show significant differences with respect to FI, indicating that palatability was not affected by the inclusion of a vegetable and animal proteins blend, and may evidence an attempt by fish to adjust the digestible energy intake. In fact, it is assumed that, up to a certain level, animals can adjust FI to meet their digestible energy needs (Boujard and Médale, 1994; Cho and Kaushik, 1985; Peres and Oliva-Teles, 1999; Yamamoto et al., 2000). On some occasions, animal protein sources can give fish palatability problems, as is the case of Laporte (2007) who evidenced that palatability of poultry meat meal, could be one of the principal factors that restricts the inclusion of this product in the diet of gilthead seabream. In contrast, in this study, the inclusion of IPM did not appear to affect negatively on the diets' palatability, similar to previous findings (Moutinho et al., 2017b) with the inclusion of MBM. Animal by-products have a positive effect on animal performance because they contain short peptides and certain AA (taurine, glycine, Arg, glutamic acid and alanine) that are stimulants for feeding and enhancers of palatability and increase acceptance of artificial diets (Martínez-Alvarez et al., 2015).

Significant differences were found in the CF between the control diet (FM100) and the FM0 diet, but no significant differences were found for the other biometric indexes (VSI, HIS and VFI). Similar results were obtained by Sánchez-Lozano et al. (2009), where even without having significant differences, there has been a slight increase in visceral fat in

seabream fed the diets that contained a greater substitution of FM. However, Kaushik et al. (2004) found a considerable increment in the amount of fat with increasing levels of FM substitution in diets for European seabass, *Dicentrarchus labrax*. Accordingly, this resulted in a similar increase in content energy of the whole body. Kaushik et al. (2004) pointed out that the high fat and energy retention values in fish fed diets with PP sources, clearly suggest that there was increased lipogenesis with increasing levels of FM replacement, without any effect on nitrogen utilization. In higher vertebrates, it is known that the level and source of dietary protein, such as soybean proteins, can affect lipid deposition, influence the pattern and potential of fatty acid bioconversion, and alter the serum and liver lipids (Aoyama et al., 2000; Dias et al., 2005; Lindholm and Eklúnd, 1991; Potter, 1995; Terasawa et al., 1994).

When the FM is substituted for alternative raw materials many factors can influence the growth and FE results. Ingredients derived from agricultural products can contain ANFs that may affect animal performance. On the contrary, animal-derived meals are exempt from these ANFs, which make their use possible because they do not pose any problem, especially in carnivorous fish. Nevertheless, the AA profile of the muscle or the efficiency in PIR and EIR can be affected by substitution, as a result of a lower efficiency in apparent retention of EAA. The results show that most of the EAA apparent retention values are affected by the substitution. Essential amino acid deficiency is one of the most important issues regarding FM substitution with alternative ingredients (Kaushik and Seiliez, 2010) and unbalanced EAA levels in the diets have been reported as one of the main causes for growth depression in fish fed animal by-products based diets (García-Gallego et al., 1998; Millamena, 2002; Moutinho et al., 2017a; Xavier et al., 2014). FM replacement affects not only the relative abundance of EAA but also NEAA, and an insufficiency in NEAA results in a reduced growth rate in fish (Schuhmacher et al., 1995). It follows that there must be an optimum dietary ratio of essential to nonessential amino acids (EAA:NEAA ratio), which will achieve maximum protein utilization for growth. Few studies have been carried out to determine the potential of some of the NEAAs and the ratios between essential and nonessential amino acids in the diet (EAA/NEAA ratio) (Hughes, 1985; Mambrini and Kaushik, 1994). Gómez-Requeni et al. (2003) found that the best growth performance in seabream occurs with a diet that resembles the EAA profile and EAA/NEAA muscle ratio, when FM has been 35% replaced by plant ingredients. In this

study, the FM100 diet had an EAA/NEAA ratio of 0.97 and the fish a mean value of 0.93. In the FM0 diet the EAA/NEAA ratio decreases to 0.66 and also fish fed with this diet showed the lowest values for retention efficiency of ingested EAA, which is related to its low final body weight gain. Other authors corroborated that gilthead seabream fed with an EAA/NEAA ratio of 1:1 have better zootechnical results than with a dietary ratio of 0.8 (Gómez-Requeni et al., 2003; Kaushik and Seiliez, 2010).

Significant differences were detected in protein ingested, energy, and EAA retention efficiencies in fish fed with the assessed diets. The diets with higher percentages of retention efficiency were FM100 and FM25, whose values are similar to other research (Moutinho et al., 2017b). This shows that a FM replacement up to 75% can be achieved according to the growth and retention results. In the present study, the results obtained in the retention efficiency for Met and Arg in the FM100 diet, are quite similar (close to 30%) to those presented by Martínez-Llorens et al. (2012). The FM0 diet presented the lowest retention efficiency for all AA, which is in accordance with the growth results obtained with this diet. This detriment in retention efficiencies may be due to lower nutrient availability due to higher fiber content in FM10 and FM0 diets reducing digestibility in diets with less FM, which has already been proven in several species, including gilthead seabream (Lupatsch et al., 1997).

The EAA_{diet}/EAA_{fish} ratio of gilthead seabream fed the FM25, FM10, FM0+ and FM0 diets presented the lowest values for all the essential amino acid. In general terms, the EAA_{diet}/EAA_{fish} values are similar to those obtained by Moutinho, et al. (2017a) with the replacement of FM for MBM in diets for seabream. In both studies, the values are lower than those of Sánchez-Lozano et al. (2011) and Martínez-Llorens et al. (2012), because the ratio has been calculated with digestible EAA. If the ratio between EAA_{diet}/EAA_{fish} is lower than 1.0 for any AA, this may signify that the EAA is "deficient" in the diet, and, in contrast, if the ratio is higher than 1.0, it may signify that this AA is "in excess" (Sánchez-Lozano et al., 2011). In present work the differences between EAA_{diet}/EAA_{fish} ratio only justified the worst FM0 diet growth, this fact must be explained by amino acid efficiency, that in general was lower with this diet, possibility due to by a poor amino acid availability in fish fed with the FM0 diet, probably caused by the inflammatory

effects of vegetable diets in gut fish. *Isochrysis* may have anti-inflammatory properties because the improvement showed in growth and nutritional parameters of FM0+ diet.

5. Conclusions

Findings from this study revealed that the up to the 75% of FM replacement is possible with a vegetable and animal (IPM) proteins blend without affect to the growth performance, feed utilization efficiency and protein metabolism of gilthead seabream (*Sparus aurata* L.). In addition, the inclusion of the microalgae *I.* aff. *galbana* (T-Iso) as additive in non-fishmeal diet (FM0+) improve the growth performance and protein efficiency of seabream.

6. Acknowledgments

The authors acknowledge Marine Microalgae Biotechnology Research Group of the University of Almeria (Spain) for supplying the microalgae *Isochrysis* aff. *galbana* (T-Iso), as part of the research supported by the SABANA project of the European Union's Horizon 2020 Research and Innovation Programme (grant # 727874).

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DISCUSIÓN GENERAL

1. Resumen de los parámetros nutricionales

Los resultados sobre el crecimiento y los índices de eficiencia nutricional obtenidos al final de los diferentes experimentos aparecen resumidos en la Tabla 1:

Tabla 1. Crecimiento y parámetros nutritivos en doradas y truchas alimentadas con piensos con altas sustituciones de harina de pescado.

	Piensos	P_i	P_f	TCI	ICA
Sust. HP por mezcla vegetal (Capítulo 1) *Oncorhynchus mykiss*	FM20	13,13	72,24^{a}	2,22^{a}	1,01^{a}
	FM10	13,43	72,94^{a}	2,20^{a}	1,09^{a}
	FM0	13,60	49,07^{b}	1,66^{b}	1,52^{b}
Inclusión aditivos en dietas 100% vegetales (Capítulo 2) *Sparus aurata*	FM100	9,50	152,00^{a}	2,09^{a}	1,57
	AA0	12,65	99,73^{b}	1,54^{d}	1,82
	N250	10,57	109,08^{b}	1,74^{c}	1,62
	N500	10,61	105,80^{b}	1,72^{c}	1,69
	P1	9,87	115,20^{b}	1,84^{b}	1,58
	P2	10,29	95,44^{b}	1,66^{c}	1,75
Sust. HP por mezcla vegetal-animal y adición de microalga (Capítulo 3) *Sparus aurata*	FM100	63,14	214,67^{a}	1,39^{a}	1,45^{a}
	FM25	64,07	193,20^{a}	1,24^{ab}	1,47^{a}
	FM10	64,09	162,90^{ab}	1,05^{b}	1,74^{ab}
	FM0	65,41	137,45^{b}	0,85^{c}	2,09^{b}
	FM0+	63,43	175,32^{ab}	1,16^{b}	1,57^{a}

P_i, peso inicial; P_f, peso final; TCI, tasa de crecimiento instantáneo; ICA, índice de conversión del alimento. Los datos se presentan como media ± SEM (n = 3). Letras diferentes en cada columna denotan diferencias significativas ($p < 0,05$) test de Newman-Keuls. Se muestra en azul el pienso con un óptimo crecimiento y en rojo el pienso con un crecimiento deficiente. Piensos Capítulo 1: FM10 y FM0, uso de gluten de trigo y torta de soja. Piensos Capítulo 2: inclusión de MPH 1% y 2%, P1 y P2; NT 250 y 500 ppm, N250 y N500. Piensos Capítulo 3: FM25, FM10, FM0 uso de harinas vegetales y HcI; FM0+ uso de harinas vegetales, HcI e inclusión microalga *I.* aff. *galbana* (T-Iso).

De acuerdo con los resultados obtenidos de la presente tesis, los piensos para doradas con un 100% de PV e inclusión de MPH y NT, presentaron resultados similares en el P_f y en el ICA, pero el pienso P1 (1% de MPH) obtuvo un TCI estadísticamente mejor que los otros y un ICA muy cercano al de la dieta control FM100. Yang et al. (2022) también encontraron buenos resultados cuando incluyeron MPH al 3% en piensos para mero híbrido (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂), ya que la ganancia en peso no fue significativamente diferente del grupo control. Los mejores resultados de crecimiento se presentaron en la dieta FM25 (ensayada en doradas) y FM10 (ensayada en truchas), las cuales contenían una sustitución de HP del 75% por una mezcla de proteína vegetal y animal (HcI), y un 90% por una mezcla vegetal, respectivamente. Estos resultados se corresponden a los encontrados por Moutinho et al. (2017) en doradas, cuando la ganancia en peso no fue significativamente diferente de la dieta control, en el

momento en que se incluyó en un 50% un subproducto de origen animal (harina de carne y huesos), y a los obtenidos por Lee et al. (2019) en truchas, quienes observaron que para un crecimiento máximo se requería HP en un nivel dietético superior al 5%. Habría que resaltar la notable mejora del crecimiento, cuando se incluyó la microalga *I.* aff. *galbana* T-Iso en el pienso con sustitución total de HP (FM0+) para doradas, respecto a los resultados obtenidos con la dieta FM0, lo que indica claramente que la inclusión de *I.* aff. *galbana* (T-Iso) tiene un efecto positivo al mismo nivel de sustitución.

En las Tablas 2 y 3 se recogen los resultados obtenidos en estudios anteriores llevados a cabo en doradas y truchas con diferentes niveles de sustitución de HP por fuentes proteicas vegetales. En términos de crecimiento, el nivel óptimo de sustitución en doradas que se alcanzó en dichos estudios, sin que éste se viese afectado, estuvo entre el 50–75%, sustituciones iguales o inferiores a las de la presente tesis doctoral, donde la utilización de MPH en una dieta basada 100% en PV y de una mezcla de PV y animal (HcI), permitió la sustitución total (Capítulo 2) y hasta un 75% de HP (Capítulo 3), respectivamente. Por otro lado, en términos de supervivencia, los estudios realizados por Estruch et al. (2018, 2015) en doradas, indicaron que una sustitución total de HP provoca un efecto negativo en la tasa de supervivencia, mientras que en esta tesis doctoral el porcentaje medio de supervivencia fue del 83% y del 96% en los Capítulos 2 y 3, respectivamente. En el caso de las truchas, el máximo nivel de sustitución alcanzado fue del 60%, sustituciones muy inferiores a la obtenida en esta tesis doctoral, donde se logró sustituir la HP en un 90% con PV sin afectar el crecimiento de los peces.

Tabla 2. Resultados obtenidos en experimentos previos en doradas alimentadas con diferentes niveles de sustitución de HP por una mezcla de fuentes proteicas vegetales.

Autores	% Sustitución	P_i	P_f	TCI	EA	S
Gómez-Requeni et al., 2004	0	16,40	$73,80^{d}$	$1,86^{c}$	$0,89^{b}$	
	50	16,70	$69,70^{c}$	$1,76^{b}$	$0,99^{a}$	
	75	16,50	$66,50^{b}$	$1,72^{b}$	$1,03^{a}$	
	100	16,40	$58,20^{a}$	$1,56^{a}$	$0,99^{a}$	
Sitjà-Bobadilla et al., 2005	0	16,40	$200,50^{d}$	$1,66^{c}$	$0,85^{a}$	
	50	16,70	$190,90^{c}$	$1,62^{bc}$	$0,95^{b}$	
	75	16,50	$181,40^{b}$	$1,59^{b}$	$0,98^{b}$	
	100	16,40	$123,70^{a}$	$1,30^{a}$	$0,84^{a}$	

De Francesco et al., 2007	0	99,10	431,40	0,44	0,77[b]	
	75	99,70	427,10	0,43	0,83[a]	
Estruch et al., 2015	0	130	393,00	0,72		88,0[a]
	100	130	360,00	0,69		60,0[b]
Estruch et al., 2018	0	131,20	393,10	0,72		88,3[a]
	100	127,20	360,40	0,69		60,0[b]

P_i, peso inicial; P_f, peso final; TCI, tasa de crecimiento instantáneo; EA, eficiencia alimenticia; S, supervivencia.

Tabla 3. Resultados obtenidos en experimentos previos en truchas alimentadas con diferentes niveles de sustitución de HP por fuentes proteicas vegetales.

Autores	Fuente proteica	% Sustitución	P_i	P_f	TCI	ICA
Yang et al., 2011	Harina de soja	0	4,02	17,43[a]	1,63[a]	1,29[b]
		20	4,02	19,56[a]	1,76[a]	1,19[b]
		40	4,01	18,19[a]	1,67[a]	1,24[b]
		60	4,02	16,93[a]	1,60[a]	1,31[b]
		80	4,00	13,60[b]	1,36[b]	1,56[a]
Brinker and Reiter, 2011	Proteína vegetal	0	86,90	167,00	1,23[a]	0,81[ab]
		50	86,10	157,20	1,16[bc]	0,89[cd]
		100	82,10	143,60	1,07[d]	0,97[e]
Hosseini Shekarabi et al., 2021	Concentrado de proteína de maíz	0	100,47	359,80[a]	2,28[a]	1,25[b]
		3	101,00	360,37[a]	2,29[a]	1,24[b]
		6	100,33	365,70[a]	2,31[a]	1,23[b]
		9	100,73	362,00[a]	2,28[a]	1,25[b]
		12	100,35	336,90[b]	2,16[b]	1,34[a]

P_i, peso inicial; P_f, peso final; TCI, tasa de crecimiento instantáneo; ICA, índice de conversión del alimento.

Durante las últimas dos décadas, la transición hacia el uso de ingredientes de origen vegetal para reducir la dependencia de la HP y el AP ha sido un desafío (Gatlin III et al., 2007; Glencross et al., 2020). De hecho, los altos niveles de sustitución en los alimentos para peces carnívoros pueden provocar efectos fisiológicos adversos sobre la digestibilidad, así como sobre la utilización de nutrientes, el crecimiento, el metabolismo, la integridad intestinal, la respuesta inmune, la resistencia a las enfermedades y la salud y el bienestar en general (Aragão et al., 2022; Colombo, 2020). Donadelli et al. (2024) y Randazzo et al. (2021) encontraron que las doradas alimentadas con piensos con fuentes vegetales presentaban un bajo crecimiento, grandes áreas de infiltración de grasa peripancreática, notables alteraciones morfológicas en el intestino y sobreexpresión de

genes relacionados con la respuesta inmune del intestino. De manera similar, Hang et al. (2022) y Jalili et al. (2013) encontraron que altas inclusiones de proteínas vegetales en piensos para trucha arco iris causan efectos indeseables sobre el crecimiento, índices nutricionales y ocasionan problemas en la salud hepática.

2. Análisis económico

2.1. ICE, IBE, IBEst

La rentabilidad económica es un aspecto muy importante a tener en cuenta a la hora de incluir diferentes materias primas en los piensos, ya que ésta es el objetivo final de las granjas acuícolas, siendo muy importante el coste que representa la alimentación de peces, pues puede suponer más del 50% de los costes de producción (FAO, 2007). Para evaluar las dietas desde el punto de vista económico, se pueden considerar tres parámetros: el índice de conversión económico (ICE) (Martínez-Llorens et al., 2007a), el índice de beneficio económico (IBE) (Martínez-Llorens et al., 2008, 2007b) y el índice de beneficio económico estándar (IBEst) (Martínez-Llorens et al., 2012). El ICE indica el coste necesario para engordar 1 kg de pescado, el IBE indica el incremento del valor añadido producido por el engorde de un pez del tamaño alcanzado en el periodo de tiempo alimentado en el experimento y el IBEst indica el incremento del valor añadido de engordar un pez considerando el gasto realizado por un pienso en concreto en un periodo determinado de tiempo de 100 días, que se calcula con el objetivo de poder comparar con otras especies, o bien se puede emplear para comparar diferentes experimentos que tienen diferente duración, de manera que la variable tiempo de engorde se unifica. Mientras que el ICE depende del precio del pienso, el IBE parece ser más apropiado a la hora de comparar la rentabilidad económica, pues considera el efecto de índices de crecimiento y la conversión del alimento, y las variables económicas, como el precio de mercado del pescado y coste del alimento. El precio de las materias primas utilizadas en los diferentes piensos experimentales se presenta en la Tabla 4.

Tabla 4. Precio de las materias primas usadas en las dietas experimentales.

Ingredientes (g kg^{-1})	Precio €/kg
Harina de pescado	1,53
Harina de trigo	0,29
Gluten de trigo	0,76
Harina de haba	0,13
Harina de soja	0,54
Harina de guisante	0,65
Harina de girasol	0,19
Aceite de pescado	1,52
Aceite de soja	1,44
Lecitina de soja	1,50
Mezcla de vitaminas y minerales	75,00
Fosfato de calcio	0,35
Taurina	4,50
Metionina	3,50
Lisina	3,00
Arginina	2,50
Treonina	3,00
Valina	2,00
Nucleótidos	75,00
Mucosa porcina hidrolizada	75,00
Harina de cerdo ibérico	1,38
Microalga *I.* aff. *galbana* (T-Iso)	37,50

Precios basados en la información suministrada por los diferentes proveedores del sector ubicados en distintas zonas de España.

Los cálculos del ICE, IBE e IBEst se realizaron aplicando las fórmulas siguientes:

$$\text{ICE } (€/\text{kg}) = \text{ICA} \times \text{Precio pienso } (\frac{€}{\text{kg}})$$

$$\text{IBE } (€/\text{pez}) = (\text{Pf} \times \text{PV}) - (\text{ICE} \times \text{Inc. peso})$$

$$\text{IBEst } (€/\text{pez} \times 100 \text{ días}) = 100 \times \frac{\text{Inc. peso}}{\text{días}} \times (\text{PV} - \text{ICE})$$

Donde ICA es el índice de conversión del alimento, Pf es el peso final de los peces y PVes el precio de venta del pescado en el mercado. El precio de venta de la dorada se calcula en 8,03 € kg^{-1} y el de la trucha en 7,81 € kg^{-1}, teniendo en cuenta el precio de éstas en algunos supermercados de cadena en Valencia y tiendas *online* a nivel nacional. El precio

de cada pienso se determinó multiplicando los respectivos aportes de cada uno de los ingredientes del alimento por su respectivo coste por kg (promedio año 2023) y sumando los valores obtenidos para todos los ingredientes en cada una de las dietas formuladas. Los resultados de estos índices se resumen en la Tabla 5.

El precio de los distintos piensos evaluados en la presente tesis doctoral, presentaron un comportamiento de disminución a medida que la sustitución de HP aumentaba (Tabla 5), con excepción de los piensos en los que se incluyó la MPH y la microalga. Si realizamos una simulación de reducción de precios para estos aditivos, los precios de los piensos P1, P2 y FM0+ bajarían haciéndolos rentables. De acuerdo a lo anterior, si el precio de la MPH fuese de 3 € kg^{-1}, el precio del pienso P1 sería de 1,58 € kg^{-1} y el del pienso P2 sería de 1,61 € kg^{-1}, llegando a tener básicamente el mismo precio que los piensos N250 y N500. En el caso de la microalga *I.* aff. *galbana* (T-Iso), si su precio fuese de 1,80 € kg^{-1}, el precio del pienso FM0+ sería de 1,74 € kg^{-1} (igual que el pienso FM25), pero si fuese un 50% más económico, es decir, si el precio de la microalga fuese de 0,90 € kg^{-1}, el precio de éste sería de 1,69 € kg^{-1} (mismo precio que el pienso FM10). La sustitución de HP por fuentes proteicas alternativas reduce los costes de los piensos (Martínez-Llorens et al., 2012; Sánchez Lozano et al., 2007), debido al elevado precio de ésta en comparación con las fuentes proteícas vegetales, lo cual es significativamente más rentable en términos económicos.

Durante los últimos 12 años, los precios europeos de la HP han aumentado de media un 37% y los del AP han aumentado de media un 85%. El nivel de precios de estos dos insumos sigue en gran medida los precios mundiales, que dependen en su mayor parte de la producción en América del Sur (Perú). Por lo que respecta al consumo de HP en la UE, éste disminuyó alrededor de un 40% entre 2009 y 2020, alrededor de 450.000 toneladas (EUMOFA, 2021). En el informe "La harina y el aceite de pescado: Producción y flujos comerciales en la UE" publicado por EUMOFA (2021), se detalla que la HP y el AP son recursos limitados y que se utilizarán cada vez más como ingredientes estratégicos en concentraciones más bajas y para etapas específicas de la producción acuícola. Además, el estudio proyecta que la producción de HP y de AP crecerá moderadamente en los próximos años, debido a la mejor utilización de los subproductos de la industria de procesamiento de pescado, y el desarrollo de otras fuentes de materias primas como el

krill, algas e insectos. De acuerdo a lo anterior, el coste de los piensos acuícolas mantendrá una tendencia en paralelo al alza o a la baja, dependiendo del precio de la HP en el mercado.

Tabla 5. Índices económicos obtenidos en los tres experimentos de la tesis.

	Piensos	**Precio pienso (€ kg^{-1})**	**ICE (€ kg^{-1})**	**IBE (€ pez^{-1})**	**IBEst (€ pez^{-1} × 100 días)**
Sust. HP por mezcla vegetal (Capítulo 1)	FM20	1,66	1,68^{b}	0,46^{b}	0,47^{b}
	FM10	1,61	1,75^{b}	0,46^{b}	0,47^{b}
	FM0	1,58	2,45^{a}	0,30^{a}	0,25^{a}
	p-valor		0,0029	0,0002	0,0001
Inclusión aditivos en dietas 100% vegetales (Capítulo 2)	FM100	1,93	3,04^{c}	0,79^{c}	0,53^{c}
	AA0	1,56	2,84^{c}	0,55^{b}	0,34^{b}
	N250	1,58	2,56^{c}	0,63^{b}	0,40^{b}
	N500	1,60	2,70^{c}	0,60^{b}	0,38^{b}
	P1	2,30	3,64^{b}	0,54^{b}	0,34^{b}
	P2	3,05	5,32^{a}	0,32^{a}	0,18^{a}
	p-valor		0	0,0001	0
Sust. HP por mezcla vegetal-animal y adición de microalga (Capítulo 3)	FM100	1,93	2,80^{ab}	1,30^{b}	0,90^{b}
	FM25	1,74	2,55^{c}	1,23^{b}	0,82^{b}
	FM10	1,69	2,94^{ab}	1,02^{ab}	0,58^{ab}
	FM0	1,67	3,49^{b}	0,85^{a}	0,37^{a}
	FM0+	3,52	5,52^{a}	0,79^{a}	0,32^{a}
	p-valor		0	0,0077	0,0032

ICE, índice de conversión económico; IBE, índice de beneficio económico; IBEst, índice de beneficio económico estándar. Los datos se presentan como media ± SEM (n = 3). Letras diferentes en cada columna denotan diferencias significativas (p < 0,05) test de Newman-Keuls. Se muestra en azul el pienso con una mejor rentabilidad económica (↓ICE ↑IBE).

En cuanto a la eficiencia económica (Tabla 5), la reducción en el coste de los piensos para truchas del Capítulo 1, de 1,66 a 1,58 € kg^{-1}, no compensó el alto ICA y el menor crecimiento, debido a que los valores del ICE y del IBE (2,41 € kg^{-1} y 0,30 € pez^{-1}, respectivamente) fueron los peores en la sustitución total de HP (FM0), por lo que no es rentable alimentar con esta dieta y tampoco genera el retorno económico deseado. Aun así, la dieta FM10 logró un IBE (0,46 € pez^{-1}) estadísticamente igual al de la dieta control FM20, lo que conlleva al retorno económico esperado. Con relación a la reducción del coste de los piensos para doradas relacionados con la sustitución total de HP (Capítulo 2) de 1,93 a 1,56 -1,60 € kg^{-1} sí compensó un mayor ICA, ya que resulta más económico alimentar con los piensos con NT y la dieta AA0 (100% vegetal), pero dicha reducción en el coste no compensó el menor crecimiento, ya que el retorno económico reflejado por

el IBE no es estadísticamente bueno. Respecto a reducción en el coste de los piensos para doradas del Capítulo 3, de 1,93 a 1,74 € kg^{-1} (dieta FM25), podemos apreciar que de acuerdo al ICE es más rentable alimentar con este pienso y que el retorno económico reflejado por el IBE de esta dieta (1,23 € pez^{-1}) fue numéricamente el segundo mejor valor y cercano al de la dieta control FM100.

Si se comparan los valores del IBE obtenidos en otros trabajos llevados a cabo con doradas, tenemos que Martínez-Llorens et al. (2007a) y Sánchez Lozano et al. (2007) encontraron que los mejores resultados del IBE (1,28 y 1,43 € pez^{-1}, respectivamente) se presentaron cuando se incluyeron PV en niveles bajos, como la harina de soja (20%) y harina de girasol (12%), respectivamente. Por el contrario, los resultados encontrados en la presente tesis indicaron que el IBE en la dieta N250 (Capítulo 2), formulada con 100% PV y 250 ppm de NT, fue numéricamente el más alto (0,63 € pez^{-1}) en relación a los demás piensos con sustitución total de HP. Por otro lado, Moutinho et al. (2017) encontraron un bajo valor del IBE (0,32 € pez⁻1) cuando sustituyeron la HP por harina de carne y huesos (HCH) en un nivel del 75%. En cambio, el pienso FM25 (Capítulo 3), con una inclusión del 75% de una mezcla de PV y HcI, presentó un alto valor del IBE (1,23 € pez^{-1}). A pesar de que estos dos piensos, son similares en el hecho de tener un 75% de PAP, presentaron diferencias notorias en el IBE, debido a que los índices de crecimiento en el pienso FM25 fueron más altos y por ende, mejores que los del pienso con 75% de HCH.

Para predecir el comportamiento del IBEst, se calcularon varios IBEst en función del precio de la dorada y la trucha en algunos supermercados de cadena en Valencia y tiendas *online* a nivel nacional: Mercadona, Consum, Carrefour, El Corte Inglés, Makro, Los Frescos del Barrio, Peix a casa, Tu Pescadería, Pescaderías Los Madrileños (Figuras 1 y 2). La recta de regresión obtenida en las gráficas de dispersión nos indica, tanto para truchas como para doradas, que existe una relación directa entre estas dos variables, es decir, que si aumenta el precio del pescado, también aumentará el IBEst. Los rangos de precio de venta de la dorada se calculan entre 7,59 y 14,36 € kg^{-1} y el de la trucha entre 7,54 y 15,83 € kg^{-1}.

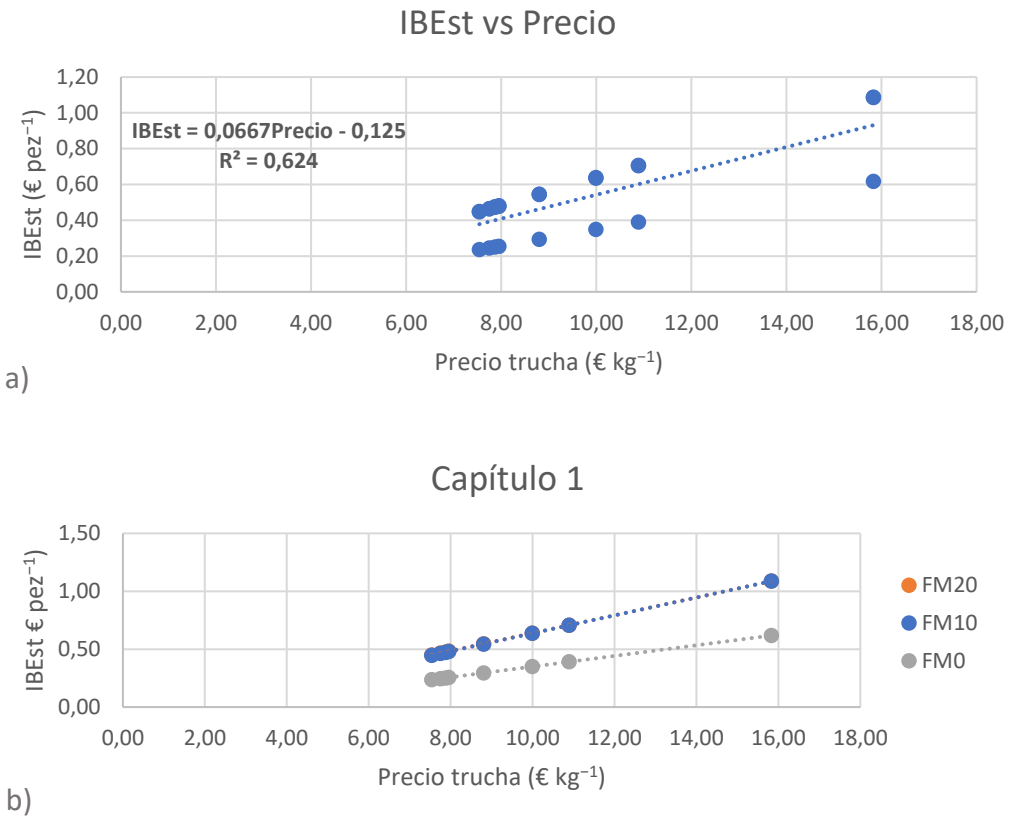
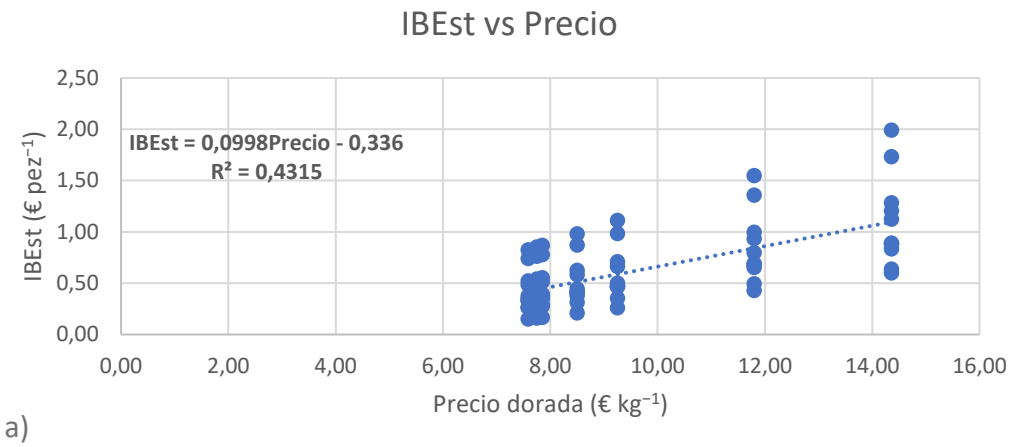
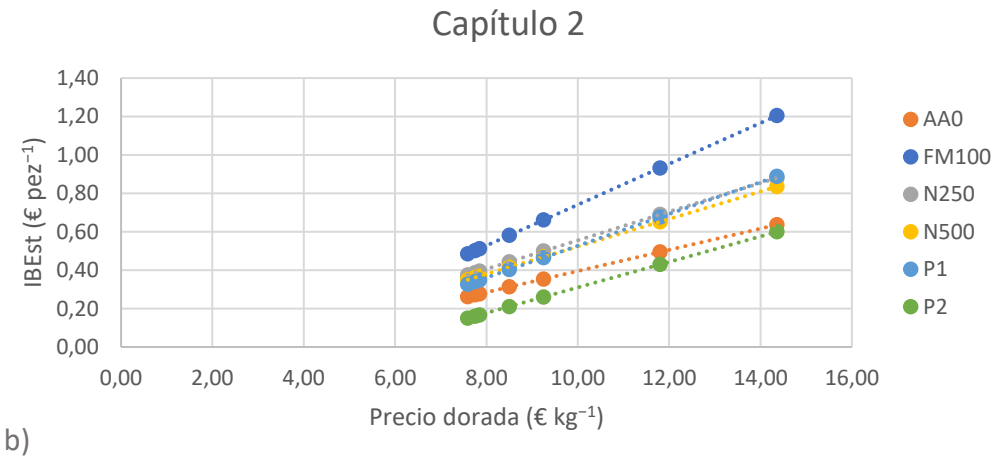


Figura 1. Índice de beneficio económico estándar (IBEst) en función del precio de la trucha. a) IBEst general calculado en función del precio de la trucha; b) IBEst calculado para los piensos del Capítulo 1.



a)



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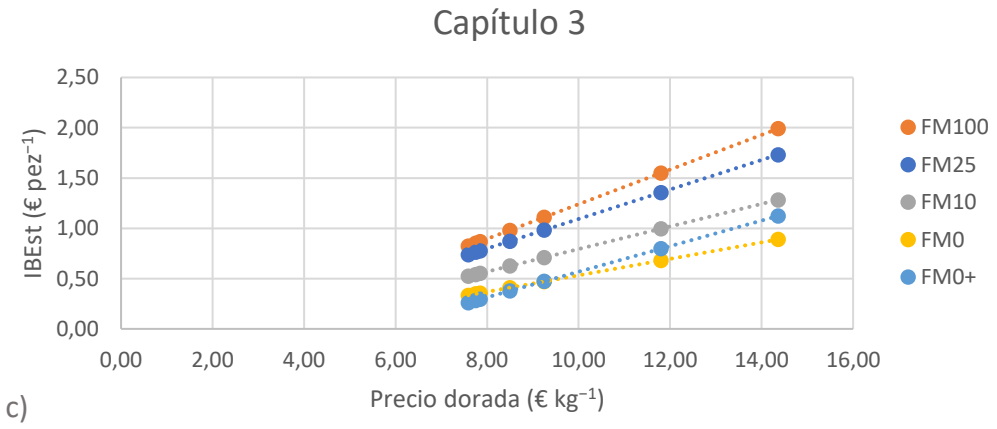

c)

Figura 2. Índice de beneficio económico estándar (IBEst) en función del precio de la dorada. a) IBEst general calculado en función del precio de la dorada; b) IBEst calculado para los piensos del Capítulo 2; c) IBEst calculado para los piensos del Capítulo 3.

Como se mencionó anteriormente, el IBEst considera el incremento de peso, la tasa de conversión alimenticia, el coste de la dieta y el precio de venta del pescado, pero para un periodo concreto de días de engorde (100 días). Es una herramienta útil si se consideran pruebas en las que el engorde se ha realizado en etapas similares con pesos iniciales similares, pero que la duración no es exactamente la misma, lo que podría influir en el incrementeo de peso, si no se estandarizarían los resultados. Se puede observar que existe una relación directa entre éste y el precio del pescado, donde el aumento del precio de venta de las doradas y truchas, provocará también un aumento del IBEst. El IBEst nos indica el retorno económico esperado y cuanto más alto sea su valor, el precio mínimo de venta del pescado será mayor. En general, se puede observar que a precios bajos de venta las diferencias entre tratamientos se minimizan, pero cuando se incrementa el precio de venta de los peces, el IBE se vuelve más significativo entre los diferentes tratamientos a favor de los piensos que contienen más HP.

Teniendo en cuenta las ecuaciones obtenidas en la regresión lineal de cada uno de los piensos evaluados, se calculó el precio mínimo de venta de la dorada y de la trucha, cuando el IBEst = 0 y el IBEst = valor máx. calculado. Estos resultados se consolidan en la Tabla 6.

Tabla 6. Precio mínimo de venta (€ kg^{-1}) de truchas y doradas de acuerdo a los valores del IBEst.

		Precio mínimo de venta (€ kg^{-1})	
		IBEst = 0	**IBEst = Máx. calc.**
Truchas (Capítulo 1)	FM20	1,67	7,79
	FM10	1,75	7,83
	FM0	2,41	7,83
Doradas Aditivos (Capítulo 2)	AA0	2,84	8,10
	FM100	3,04	8,02
	N250	2,56	8,06
	N500	2,70	8,01
	P1	3,64	7,99
	P2	5,33	8,04
Doradas Ibérico (Capítulo 3)	FM100	2,80	8,03
	FM25	2,55	8,01
	FM10	2,93	8,01
	FM0	3,49	8,02
	FM0+	5,53	8,05

Así, se puede obtener para cada uno de los piensos, cuál es el precio mínimo de venta, sin considerar cualquier otro coste. Por ejemplo, en el pienso AA0, cuando el IBEst = 0 el precio mínimo de venta de la dorada sería de 2,84 € y si el IBEst = 0,29 el precio mínimo de venta sería de 8,10 €.

2.2. Análisis de sensibilidad

El análisis de sensibilidad es una herramienta que nos permite estudiar cómo afectan los distintos valores de una variable independiente a una variable dependiente concreta en ciertas condiciones específicas. Para determinar cómo afecta el precio de la HP sobre el precio del pienso, el ICE y el IBEst, se simularon varios escenarios en los que el precio de la HP presentaba una tendencia al alza y el precio de los aditivos, HcI y fuentes proteicas vegetales utilizados en los piensos, presentaba una tendencia a la baja. Los piensos FM10 (Capítulo 1), P1 (Capítulo 2) y FM25 (Capítulo 3) se tuvieron en cuenta a la hora de presentar las gráficas, ya que éstos fueron los que exhibieron los mejores rendimientos en cuanto a parámetros nutricionales se refiere (Figura 3). El precio de las materias primas causa un gran efecto sobre el precio de los piensos y es que la reducción de éste, conlleva a una disminución en el precio de los piensos P1 y FM10 en un 13% y 21%, respectivamente, pero produce un aumento en el precio del pienso FM25 en un 7%. Los precios mínimos de las materias primas que se tuvieron en cuenta en la simulación de los diferentes escenarios de variación de precios con tendencia a la baja, fueron de 40.000 € g^{-1} para la MPH, de 200 € g^{-1} para las fuentes proteicas vegetales y de 800 € g^{-1} para la HcI.

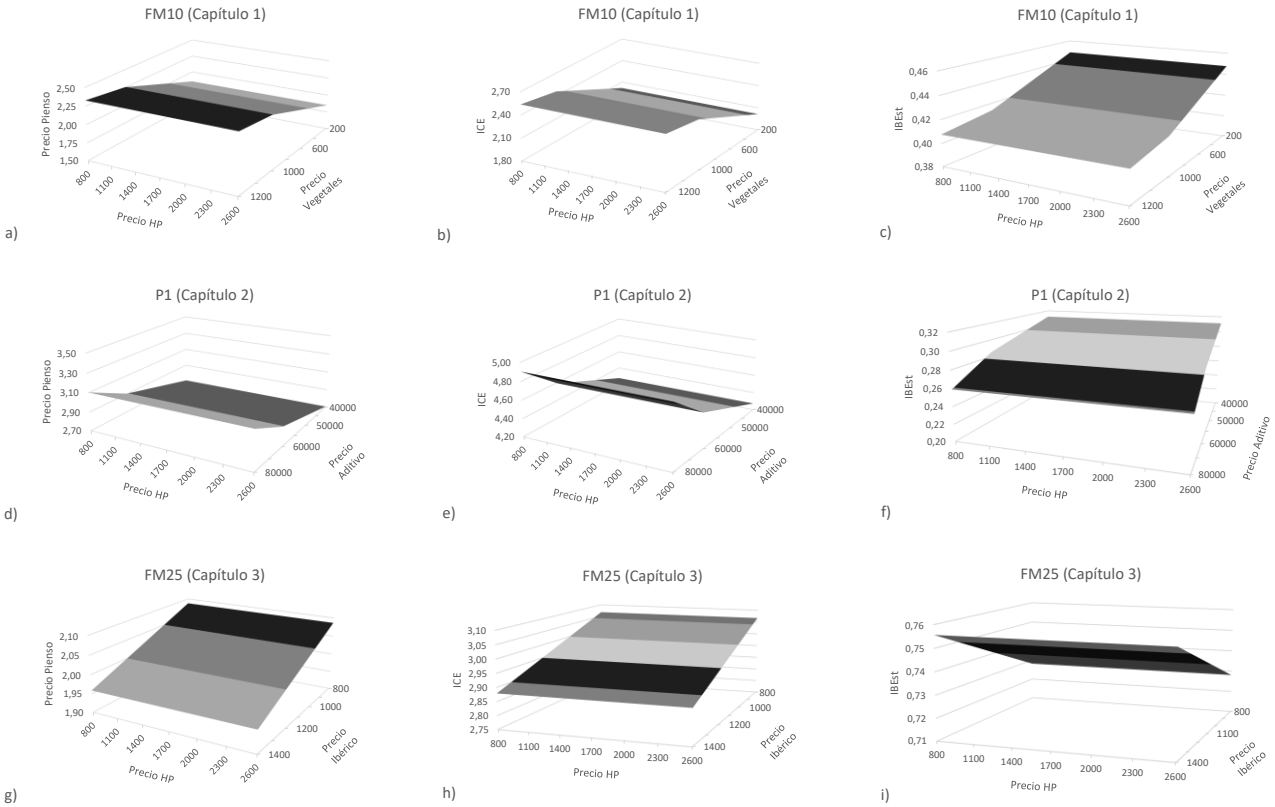

Figura 3. Influencia de las variaciones del precio de la HP e ingredientes clave utilizados en los piensos experimentales sobre el precio del pienso, el ICE y el IBEst. a), b) y c) variaciones sobre el pienso FM10 del Capítulo 1; d), e) y f) variaciones sobre el pienso P1 del Capítulo 2; g), h) e i) variaciones sobre el pienso FM25 del Capítulo 3.

3. Análisis ambiental de la sustitucion de la harina de pescado en piensos

3.1. Índice FIFO

El índice FIFO (pescado requerido:pescado obtenido) o *Fish In:Fish Out* por su sigla en inglés, expresa cuántos kilos de pescado silvestre se necesitan para producir un kilo de pescado y se desarrolló como una forma de examinar el uso de ingredientes marinos durante la producción animal, aunque la mayor parte del enfoque se ha centrado en la acuicultura en relación con el uso de peces silvestres en los alimentos balanceados para la producción de peces. FIFO se utiliza a menudo como referencia medioambiental simplificada y, aunque sigue siendo una métrica importante, tiene limitaciones. Estos incluyen su aplicación a un sector/ingrediente en forma aislada de una contribución más global, especialmente el valor y la calidad de los ingredientes. FIFO no distingue entre subproductos y fuentes de pesquerías de reducción. Sustituir la harina y el aceite de

pescado no implica necesariamente que esos otros recursos sean más sostenibles o se obtengan de manera responsable (IFFO, 2022).

El índice FIFO se calcula de la siguiente manera:

$$\text{FIFO} = \text{ICA} \times \left(\frac{\%\ \text{HP pienso} + \%\ \text{AP pienso}}{\text{RH} + \text{AH}}\right)$$

Donde ICA es el índice de conversión del alimento, % HP pienso es el porcentaje de inclusión de harina de pescado, % AP pienso es el porcentaje de inclusión de aceite de pescado, RH es el rendimiento de la harina de pescado y RA el rendimiento del aceite de pescado (IFFO, 2020). Según Tacon y Metian (2008), el rendimiento de la harina y del aceite de pescado que se obtiene de la pesca pelágica es de un 22,5% y de un 5%, respectivamente. Esto quiere decir, que de 1000 kg de peces se obtienen entonces 225 kg de harina de pescado y 50 kg de aceite.

En general, existe una continua reducción en los índices FIFO, reflejando el uso de HP y AP como ingredientes estratégicos en puntos clave de los ciclos productivos de la acuicultura con tendencia a optimizar sus aportes nutricionales. La cifra general de acuicultura alimentada muestra una marcada disminución a 0,19 (Tabla 7), lo que esencialmente significa que, por cada 0,19 kg de pescado salvaje entero utilizado en la producción de HP, se produce un kilo de pescado de piscifactoría. Es decir, por cada kg de pescado salvaje utilizado se producen 5 kg de pescado de piscifactoría. Ha habido una reducción en todas las categorías, pero es de particular interés la cifra de salmónidos, que para 2020 se considera inferior a 1,0, es decir, la industria de piensos para salmónidos apoya la producción de más peces de piscifactoría de los que utiliza como alimento para peces (IFFO, 2020).

Tabla 7. Reducción de las cifras FIFO durante las últimas dos décadas

Especies	2000	2010	2020
Peces marinos	2,21	0,98	0,75
Salmónidos	3,03	1,87	0,93
Producción total de acuicultura	0,47	0,28	0,19

Fuente: IFFO, 2020

Los índices FIFO obtenidos en la presente tesis se muestran en la Tabla 8. Considerando que en 2020 el índice FIFO para peces marinos y salmónidos se situó en 0,75 y 0,93, respectivamente, y teniendo en cuenta los piensos que proporcionaron los mejores resultados respecto a los parámetros de crecimiento, de aprovechamiento nutritivo y valores económicos, se puede apreciar que los piensos FM10 del Capítulo 1, P1 del Capítulo 2 y FM0+ del Capítulo 3 se ajustan a la tendencia de optimización del uso de HP y AP, aunque en conjunto, todos los piensos excepto el FM20 (Capítulo 1) y los FM100 (Capítulos 2 y 3), encajan en dicha tendencia.

Tabla 8. Índices FIFO obtenidos con las dietas evaluadas en la presente tesis doctoral.

	Pienso	**HP (g kg^{-1})**	**AP (g kg^{-1})**	**Índice FIFO**
Sust. HP por mezcla vegetal (Capítulo 1)	FM20	200	82	1,04^{a}
	FM10	100	91	0,75^{b}
	FM0	0	100	0,55^{c}
Inclusión aditivos en dietas 100% vegetales (Capítulo 2)	FM100	589	38	3,58^{a}
	AA0	0	78	0,52^{b}
	N250	0	78	0,46^{b}
	N500	0	78	0,48^{b}
	P1	0	78	0,45^{b}
	P2	0	78	0,50^{b}
Sust. HP por mezcla vegetal-animal y adición de microalga (Capítulo 3)	FM100	590	45	3,34^{a}
	FM25	150	85	1,26^{b}
	FM10	60	90	0,95^{bc}
	FM0	0	100	0,76^{c}
	FM0+	0	100	0,57^{c}

HP, harina de pescado; AP, aceite de pescado; FIFO, *fish in:fish out.* Se muestra en azul el pienso con un mejor índice FIFO teniendo en cuenta el análisis nutricional y económico.

Los resultados indican que el pienso P1 (Capítulo 2) presenta el mejor índice FIFO respecto a los demás piensos evaluados en la presente tesis doctoral, el cual estaba formulado en su totalidad con fuentes proteicas vegetales y la inclusión de 1% de MPH como aditivo. La disminución del uso de la HP y el AP en los piensos acuícolas ha suscitado una marcada reducción del índice FIFO, y por ende una mejora en la sostenibilidad de la acuicultura.

En términos generales, los índices FIFO obtenidos en esta tesis doctoral, señalan que la sustitución de la HP por otras materias primas, que a su vez garantizan los mismos valores nutricionales característicos del pescado, pero con un menor coste medioambiental y la utilización de subproductos animales (HcI) y de aditivos alimentarios como las

microalgas, NT y MPH, brindan grandes posibilidades de tener una acuicultura cada vez más sostenible.

3.2. Análisis del Ciclo de Vida

La acuicultura se encuentra entre los sectores alimentarios de más rápido crecimiento a nivel mundial (Comisión Europea, 2020a), con una tasa de crecimiento promedio del 5,3% en el período 2001–2018 (FAO, 2020). En los países mediterráneos, la producción acuícola ha crecido constantemente durante las últimas décadas, y se espera que esta tendencia continúe (Zoli et al., 2023). A pesar de que la acuicultura suele considerarse sostenible (Tlusty and Thorsen, 2017), este rápido crecimiento se asocia con frecuencia a un impacto medioambiental negativo causado por la eutrofización de los ecosistemas acuáticos, la explotación intensiva del agua y la tierra, la introducción de especies de peces alóctonas y la ecotoxicidad producida por el uso de productos químicos (Bohnes et al., 2019; Ottinger et al., 2016). Por lo tanto, la identificación de nuevas estrategias y sistemas de producción que sean sostenibles desde el punto de vista medioambiental es primordial.

En diciembre de 2019, la Comisión Europea lanzó el Pacto Verde Europeo (Comisión Europea, 2019), la nueva estrategia de crecimiento de la UE, que tiene como objetivo reducir la contaminación y las emisiones de carbono, impulsar el uso eficiente de los recursos y restaurar la biodiversidad. El Pacto Verde Europeo, la Estrategia de la Granja a la Mesa (Comisión Europea, 2020b) y las directrices estratégicas para una acuicultura de la UE más sostenible y competitiva (Comisión Europea, 2021), enfatizan el potencial de la acuicultura como un importante contribuyente a un sistema alimentario sostenible y responsable, en particular como fuente de proteína de baja huella de carbono. Por todas estas razones, en el contexto económico y social actual, el desarrollo sostenible del sistema acuícola se está convirtiendo cada vez más en una prioridad (Marvin et al., 2020). Los objetivos principales son optimizar y reducir los insumos del sistema, reducir el consumo de energía y, de manera más general, mejorar la eficiencia de todo el sistema para mejorar el desempeño ambiental del sector (d'Orbcastel et al., 2009).

Para evaluar la sostenibilidad medioambiental de los sistemas de producción de alimentos, una valiosa herramienta está representada por el análisis del ciclo de vida o *Life Cycle Assessment* (LCA), que está demostrando un papel clave en el camino hacia la

sostenibilidad de los alimentos (Ziegler et al., 2016), ya que se considera el enfoque más adecuado para analizar un amplio espectro de impactos ambientales (Guinee, 2002). LCA utiliza un enfoque completo al evaluar el efecto potencial de la acuicultura en el medioambiente, donde se consideran minuciosamente todos los factores involucrados, incluidas las materias primas, el transporte, la distribución, el uso, el mantenimiento, el reciclaje y la gestión de desechos (Sun et al., 2023). LCA es un método normalizado por la Organización Internacional de Normalización - ISO (ISO, 2006) que se puede utilizar para cuantificar los impactos de los sistemas y productos de producción en los recursos naturales, la salud humana y los ecosistemas a lo largo de todo su ciclo de vida (EC, 2010; Ghamkhar et al., 2020). El método LCA se puede aplicar para evaluar múltiples categorías de impacto y también se puede utilizar como herramienta de toma de decisiones en la industria de la acuicultura. De hecho, el LCA se ha utilizado para detectar los puntos críticos de un sistema determinado, cuyos impactos pueden reducirse posteriormente identificando alternativas novedosas o estrategias de producción menos perjudiciales (Bohnes et al., 2019).

En la presente tesis doctoral, se evaluó el impacto ambiental de la sustitución de la HP por HcI y la inclusión de la microalga *Isochrysis* aff. *galbana* (T-Iso) en piensos para doradas (Capítulo 3), basándose en evaluaciones del ciclo de vida y los resultados preliminares del potencial de calentamiento global (GWP - acrónimo del inglés *Global Warming Potential*) calculado para 1 kg de HcI y 1 kg de microalgas (peso seco) fueron:

- Harina de cerdo ibérico (HcI): 2,85 kg CO_2-eq/kg (la mayor parte del impacto ambiental se debe a la producción porcina de 145 kg de peso vivo)

- Microalga *Isochrysis* aff. *galbana* (T-Iso): 4,91 kg CO_2-eq/kg (casi el 80% del impacto ambiental se debe a la electricidad de la red).

La HcI utilizada en los piensos para doradas fue suministrada por el matadero Jamón y Salud (Llerena, Badajoz, España), la cual se elaboró a partir de subproductos de cerdos cruzados (Ibérico × Duroc, 50:50), criados en libertad en dehesas, las cuales son aprovechadas para la montanera donde los cerdos se alimentan de bellotas y pasto. La producción tradicional del cerdo ibérico se caracteriza por sistemas al aire libre que producen animales alimentados con recursos naturales. La producción de cerdo ibérico

en montanera tiene los menores impactos por cambio climático, acidificación, eutrofización y demanda energética acumulada, debido al uso estricto de los recursos naturales (bellota y hierba) durante el periodo de engorde, que cuando se basa en engordes de cebo de campo que dependen más de piensos compuestos (García-Gudiño et al., 2020). Los rendimientos de la canal para cerdos oscilan entre el 78-80%, pudiendo llegar hasta el 81-85%, siendo la media del 80%. Este incremento se debe al impacto que generan la alimentación, el sexo y la edad sobre los rendimientos productivos del cerdo (Ortiz et al., 2021; Rey et al., 2006; Serrano et al., 2008). Al-Zohairi et al. (2023) llevaron a cabo un estudio que se centró en mejorar el uso de subproductos de cerdo en la fase de matadero, donde realizaron un LCA de la producción de carne de cerdo utilizando datos reales de cuatro mataderos europeos. El estudio mostró variaciones sustanciales en la proporción de cerdo en peso vivo utilizado para alimentación humana, con un rendimiento comestible que oscila entre el 72% y el 88%. Asimismo, plantean que, con una optimización del rendimiento de la canal, en la que el 92% del peso vivo del cerdo se utilice como productos comestibles, podría reducirse el impacto ambiental por kg de producto porcino vendido para consumo humano entre un 4% y un 26%, lo cual se vería reflejado en un ahorro de emisiones de carbono entre 2,06 y 2,17 kg CO_2-eq/kg en los subproductos de cerdo utilizados como alimento para animales. Es, por lo tanto, que en este trabajo no solo se intenta reducir el impacto ambiental por la reducción del uso de HP en el pienso para peces, sino también con el uso de un subproducto que, además es apto en la formulación de alimentos con certificado ecológico, por lo que estos resultados deberían tomarse en cuenta a la hora de obtener un producto acuícola con certificación ecológica.

La microalga *Isochrysis* aff. *galbana* (T-Iso) fue suministrada por la Universidad de Almería, la cual fue producida en reactores tubulares con un consumo de electricidad proveniente de la red de 10 kWh/kg. Anteriormente se mencionaba que el 80% del impacto ambiental se debe a la electricidad y es que el consumo de electricidad es uno de los principales impulsores de los impactos ambientales durante las fases de producción de microalgas. La mayoría de los estudios de LCA de biorrefinerías de microalgas han asumido que la electricidad se proporcionaría desde la red (Barr and Landis, 2018; Batan et al., 2010; Beal et al., 2018, 2015; Sills et al., 2013). Aun así, las microalgas marinas se muestran prometedoras como posibles sustitutos de la HP y el AP en los piensos para acuicultura debido a sus elevados perfiles de AG y su alto contenido de proteínas

(Bélanger-Lamonde et al., 2018; Kiron et al., 2016; Sarker et al., 2020a, 2020b, 2018, 2016a, 2016b), así como una alternativa ambientalmente sostenible (Nagappan et al., 2021). En primer lugar, las microalgas no requieren tierra cultivable y tienen mayores rendimientos de biomasa que las plantas o animales terrestres (Benedetti et al., 2018). En segundo lugar, debido a que las microalgas marinas pueden cultivarse utilizando agua de mar o aguas residuales, requieren menos agua potable que otros productos agrícolas (Merlo et al., 2021; Moomaw et al., 2017). En tercer lugar, las microalgas tienen el potencial de reciclar dióxido de carbono (CO_2) de los gases de combustión y otras fuentes industriales (Daneshvar et al., 2022). Por lo tanto, el cultivo de microalgas marinas no compite con la producción convencional de alimentos por tierras cultivables y recursos de agua dulce y podría ser más sostenible ambientalmente con respecto al cultivo extensivo (Benedetti et al., 2018). Además, al reciclar los gases de combustión de las centrales eléctricas de energía fósil, las microalgas tienen el potencial de mitigar las emisiones de CO_2 y permitir la diversificación y expansión de productos y materias primas de origen biológico (Wilson et al., 2021).

En la Tabla 9 se presenta el rendimiento y el GWP en razón de los piensos consumidos por las doradas (Capítulo 3), y en ella se puede apreciar cómo va aumentando este último a medida que aumenta la cantidad de HcI en el pienso. Al comparar las dietas FM0 y FM0+, la cuales tenían la misma cantidad de HcI, vemos cómo disminuye el GWP en un 14% cuando se adiciona la microalga (FM0+). Esta misma tendencia la podemos apreciar en los resultados de GWP debido a los piensos consumidos, considerando la HP y el AP provenientes de residuos de pescado (Tabla 10), donde se observa un aumento similar del GWP a medida que va aumentando la cantidad de HcI en los piensos y la disminución en un 14% en la dieta FM0+ respecto a la dieta FM0.

Tabla 9. Rendimiento y potencial de calentamiento global (por kg de peso corporal ganado) debido a los piensos consumidos.

	FM100	**FM25**	**FM10**	**FM0**	**FM0+**
Peso corporal inicial (g)	63,1	64,1	64,1	65,4	63,4
Peso corporal final (g)	214,7	193,2	162,9	137,4	175,3
Peso corporal ganado (g)	151,6	129,1	98,8	72,0	111,9
ICA	1,5	1,5	1,7	2,1	1,6
kg CO_2-eq del alimento consumido/kg peso corporal ganado	1,15	2,01	2,48	3,26	2,81

Tabla 10. Potencial de calentamiento global (por kg de peso corporal ganado) debido a los piensos consumidos, considerando la harina de pescado y el aceite de pescado procedentes de residuos de pescado (en lugar de capturas).

	FM100	FM25	FM10	FM0	FM0+
kg CO_2-eq del alimento consumido/kg peso corporal ganado	1,55	2,13	2,55	3,30	2,84

Los resultados del análisis de puntos críticos, en el que evaluamos el porcentaje de contribución individual de los ingredientes para el GWP a causa del pienso consumido, se presentan en la Figura 4.

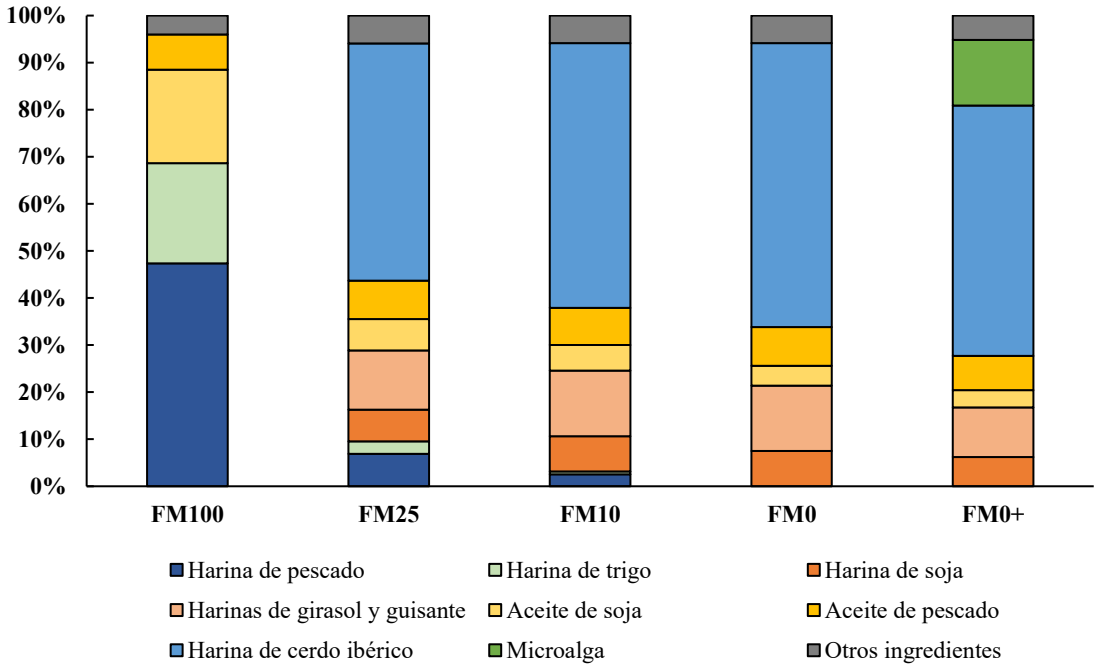

Figura 4. Análisis de puntos críticos (% de contribución debida a cada ingrediente) para el potencial de calentamiento global debido a los piensos consumidos.

La contribución del GWP de la HP fue del 47%, 7% y 3% para los piensos FM100, FM25 y FM10, respectivamente. En cuanto a la HcI, su contribución al GWP, como era de esperar, aumentó a medida que aumentaba el nivel de sustitución de la HP, siendo del 50%, 56% y 60% en las dietas FM25, FM10 y FM0, respectivamente, pero la dieta FM0+, en comparación con la dieta FM0, produjo un menor GWP (-12%). La contribución de la microalga T-Iso fue del 14%. Se identificó que los ingredientes de origen vegetal como las harinas de trigo, soja, habas y girasol, hicieron contribuciones notablemente menores

del GWP respecto a la HcI, siendo del 22% en las dietas FM25, FM10 y FM0, y del 17% en la dieta FM0+.

CONCLUSIONES

Esta tesis es una contribución al estudio de la reducción de la harina de pescado en piensos sobre el crecimiento, parámetros nutritivos y salud intestinal de las dos especies con mayor relevancia en la producción acuícola marina y continental a nivel nacional, como son la dorada y la trucha arco iris.

De los resultados obtenidos en la presente tesis doctoral se concluye:

En doradas:

1. Un 75% de sustitución de harina de pescado por una mezcla de proteína vegetal y animal, no afecta al crecimiento, parámetros nutritivos ni al metabolismo proteico.
2. La inclusión de aditivos (1% de mucosa porcina hidrolizada, 250 mg/kg de nucleótidos o 5% de la microalga *Isochrysis* aff. *galbana*, T-Iso) en piensos sin harina de pescado mejora el crecimiento y la eficiencia nutritiva.
3. Además de la mejora en los parámetros zootécnicos, los piensos sin harina de pescado y con inclusión de nucleótidos, así como el pienso con un 75% de sustitución de harina de pescado, muestran un excelente beneficio económico, lo que supone una mayor rentabilidad económica.
4. Como era esperable, los piensos sin harina de pescado reducen el índice FIFO, lo que mejora con la adición de mucosa porcina hidrolizada al 1% o de la microalga *I.* aff. *galbana.*
5. Las evaluaciones del análisis del ciclo de vida y los resultados del potencial del calentamiento global, indicaron que la harina de cerdo ibérico y la microalga *I.* aff. *galbana* generan el mayor impacto ambiental.
6. La sustitución de harina de pescado al 75% por una mezcla de proteína vegetal y animal presenta el menor potencial del calentamiento global.
7. La inclusión de microalga en piensos sin harina de pescado supone una reducción del 14% en el potencial del calentamiento global.

En truchas:

8. Es posible sustituir el 90% de la harina de pescado en piensos, utilizando una mezcla de fuentes proteicas vegetales (gluten de trigo y torta de soja), sin efectos negativos en el crecimiento o la salud intestinal.

9. Sumado a la mejora del crecimiento, con el pienso con el 90% de sustitución de harina de pescado también se optimizan los parámetros económicos y el índice FIFO.

En síntesis, la sustitución total de harina de pescado sin inclusión de aditivos no es recomendable para la alimentación de especies carnívoras como la dorada y la trucha. Sin embargo, la utilización de éstos mejora el crecimiento y la eficiencia nutritiva, y especialmente, favorece la rentabilidad económica de la alimentación y minimiza su impacto ambiental.

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