Evaluation of organic plant and animal ingredients for fish feeding

Science and Technology of Animal Production Doctoral Programme

Doctoral Thesis presented by:

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Valencia, February 2024

List of published articles:

Tefal, Eslam, Ignacio Jauralde, Ana Tomás-Vidal, Silvia Martínez-Llorens, David S. Peñaranda, and Miguel Jover-Cerdá. 2023. "New Organic Raw Materials for Gilthead Seabream (*Sparus aurata*) Feeding and the Effects on Growth, Nutritive Parameters, Digestibility, and Histology" *Fishes* 8, no. 6: 330. https://doi.org/10.3390/fishes8060330

Tefal, Eslam, Ana Tomás-Vidal, Silvia Martínez-Llorens, Ignacio Jauralde, David Sánchez-Peñaranda, and Miguel Jover-Cerdá. 2023. "Effects of Eco-Organic Feed on Growth Performance, Biometric Indices, and Nutrient Retention of Gilthead Seabream (*Sparus aurata*)" *Sustainability* 15, no. 14: 10750. https://doi.org/10.3390/su151410750.

Tefal, Eslam, Silvia Martínez-Llorens, Ana Tomás-Vidal, Ignacio Jauralde, David Sánchez-Peñaranda, and Miguel Jover-Cerdá. 2023. Organic Ingredients as Alternative Protein Sources in the Diet of Juvenile Organic Seabass (*Dicentrarchus labrax*). *Animals*, *13*(24), 3816. https://doi.org/10.3390/ani13243816

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Tefal, Eslam, David Sánchez-Peñaranda, Silvia Martínez-Llorens, Ana Tomás-Vidal, Ignacio Jauralde, and Miguel Jover-Cerdá. 2023. Effect of fishmeal substitution under organic farm conditions in rainbow trout (*Oncorhynchus mykiss*). Under review at *Aquaculture.*

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Piensos para dorada con ingredientes 100% ecológicos.
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Crecimiento de Trucha Arcoíris Alimentados con piensos ecológicos.
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Publicación: XIV Congreso Internacional de SEAE de Agricultura Ecológica, 2022 Resúmenes, páginas 202.

Evaluación Nutritiva de Ingredientes Ecológicos en Piensos para Dorada *Sparus aurata*.

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Publicación: XVIII Congreso Nacional de Acuicultura "Acuicultura: mares y ríos de oportunidades" CÁDIZ Del 21 al 24 de Noviembre 2022. Resúmenes, páginas 111-112.

Evaluación del efecto en el crecimiento y microbiota intestinal de una producción ecológica en trucha arco iris (*Oncorhynchus mykiss*).

Eslam Tefal, Ana Tomás-Vidal, Ignacio Jauralde-García, Ignacio Giménez, Jose Vicente Roig-Genoves, Andrés Moñino-López, Silvia Martínez-Llorens, Miguel Jover Cerdà, David Sanchez Peñaranda.

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XVIII Congreso Nacional de Acuicultura celebrado en Cádiz del 21 al 24 de noviembre de 2022. Oral Communication.

Publicación: XVIII Congreso Nacional de Acuicultura "Acuicultura: mares y ríos de oportunidades" CÁDIZ Del 21 al 24 de Noviembre 2022. Resúmenes, páginas 52-54.

Efecto de la alimentación con piensos ecológicos sobre la histología intestinal y hepática en lubina (*Dicentrarchus labrax*).

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XVIII Congreso Nacional de Acuicultura celebrado en Cádiz del 21 al 24 de noviembre de 2022. Oral Communication.

Publicación: XVIII Congreso Nacional de Acuicultura "Acuicultura: mares y ríos de oportunidades" CÁDIZ Del 21 al 24 de Noviembre 2022. Resúmenes, páginas 81-82.

In the depths of the natural world, where harmony and sustainability intertwine, lies a realm of endless possibilities. This doctoral thesis stands as a testament to the exploration and evaluation of organic plant and animal ingredients for the nourishment of aquatic life.

As we delve into the intricacies of this vital ecosystem, let us be guided by the profound understanding that nature's wisdom holds the key to a balanced and thriving world. With each discovery and insight gained, may we sow the seeds of a more sustainable future, where the delicate dance of life continues to flourish in harmony with our planet's precious resources.

ACKNOWLEDGMENTS

I extend my deepest gratitude to all those who have contributed to completing this doctoral thesis on evaluating organic plant and animal fish-feeding ingredients.

First and foremost, I express my heartfelt appreciation to my loving and supportive family, who have been my constant source of strength and inspiration throughout this doctoral journey. I want to acknowledge the unwavering support and sacrifices my wife, Aya Taha, made. Her patience, understanding, and encouragement have been instrumental in my pursuit of this academic milestone. Her unwavering belief in me has guided me even during the most challenging times.

I would like to express my sincere appreciation to all aquaculture and biodiversity research group members, especially my supervisors, Miguel Jover Cerdá and Ignacio Jauralde García, for their unwavering guidance, invaluable expertise, and continuous support throughout this research journey. Your mentorship and dedication have played a vital role in shaping my academic growth and pushing the boundaries of scientific exploration. Special thanks to the rest of the research group, Ana Tomás Vidal, Silvia Llorens Martínez, and David Sanchez Peñaranda, for their exceptional help, advice, support, patience, and time dedicated to me and this project.

I would like to thank Andrés Moñino for his tireless efforts in preparing the feed and caring for my fish for many years. I also want to thank Javi Moya and Luis for opening their laboratory doors to me whenever I needed assistance. Thanks to Mari Milián and Raquel Monge for their invaluable analysis assistance and willingness to help whenever needed. My heartfelt appreciation goes to all my colleagues, cafes, and others who have provided support along the way, including Sara, Marian, Ronny, and Glenda Vélez.

I must acknowledge Francisco Moyano from Almeria University, who has collaborated with and supported this work. I would also like to extend my heartfelt thanks to the members of my doctoral committee for their insightful feedback, constructive criticism, and valuable suggestions that have enhanced the quality of this thesis.

I am grateful to the Ministry of Higher Education of the Arab Republic of Egypt and Damanhour University for providing the necessary resources, facilities, and financial support to enable me to conduct this research effectively. Additionally, I appreciate the collaboration of the Biodiversity Foundation (Spanish Ministry for Ecological Transition and the Demographic Challenge) through the Pleamar Program, co-financed by the European Maritime and Fisheries Fund (EMFF).

I am indebted to my colleagues and fellow researchers, who have been a constant source of inspiration, collaboration, and intellectual stimulation. Your support and camaraderie have made this journey more rewarding and fulfilling. I also appreciate the participants and contributors involved in this study. This research would not have been possible without their cooperation and willingness to share their knowledge and experiences.

My dear friends and family, thank you for your unwavering encouragement, understanding, and love. Your support has been my pillar of strength throughout this academic pursuit.

Lastly, I would like to express my gratitude to all the individuals who may not be directly mentioned but have significantly shaped my academic and personal growth. Your contributions, no matter how big or small, have made a lasting impact on my journey.

To my beloved family, especially my wife, thank you for standing by my side, believing in my dreams, and providing unwavering support throughout this doctoral journey. Your love, patience, and sacrifices have been the driving force behind my accomplishments.

This thesis is a testament to my family's collective effort, love, and support. I am forever grateful. I sincerely thank all who have supported and believed in me. This thesis is a testament to my hard work, dedication, and the collaboration of an incredible network of individuals.

Thank you.

Eslam Tefal

ABSTRACT

The organic or ecological aquaculture is gaining popularity as consumers become more aware of sustainable and environmentally friendly food production practices. Diets with organic ingredients are an essential component of ecological aquaculture. These diets use organic ingredients and specific formulations to promote healthier and environmentally friendly aquatic animal production. They align with the principles of organic farming, minimize the use of synthetic chemicals, and aim to produce healthier and environmentally responsible seafood products.

This doctoral thesis comprises four distinct studies with the aim of advancing our understanding of the integration of new organic ingredients in aquaculture diets and their effects on various species, focusing on gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), and rainbow trout (*Oncorhynchus mykiss*). These studies explore growth, body composition, feed utilization, digestibility, histological responses, and microbiota composition when incorporating novel organic foods and ingredients.

In the first study, the effect of eco-organic feed on the growth, nutritional efficiency, feed utilization, and body composition of gilthead sea bream was investigated. Six diets were tested, including a control diet (CONT) without organic ingredients, four diets with 100% organic ingredients (rainbow trout byproduct - TRO, European sea bass byproduct - SBS, poultry byproducts - POU, and a mixture - MIX), and an organic control diet (ORG) with organic ingredients and 30% fish meal. In the 70-day experiment, fish were fed twice a day, starting with an initial weight of 60.5 g. The results revealed that the highest growth rates were observed in fish fed the ORG and CONT diets containing fish meal. In contrast, the POU diet showed the lowest growth rate, the lowest survival rate, and the highest feed conversion ratio (FCR). The efficiency of essential amino acids was generally high in fish fed ORG and CONT diets, with notable differences in fatty acid retention efficiency in all diets. The CONT diet showed the highest retention efficiency, followed by the ORG diet. However, the economic conversion rate was lower for CONT, SBS, TRO, and MIX. In summary, the use of organic diets of animal origin affected the growth performance of gilthead sea bream, with promising potential despite variations in economic conversion rates between different diets.

In the second study, the use of new organic ingredients to feed gilthead sea bream with an initial weight of 93 ± 3.82 g was investigated for 120 days, focusing on growth, nutritional parameters, digestibility, and histology. Four diets were tested: an organic control diet (CON) and three diets with 100% organic materials - rainbow trout byproduct (TRO), Iberian pork byproduct (IBE), and insect meal as a protein source (INS). At the end of

the experiment, fish fed the CON diet showed the highest weight, with no significant difference between the experimental diets. The crude protein content was higher in fish fed TRO and INS diets, while the highest crude fat value was observed in the CON diet. Fish fed IBE and INS diets demonstrated high digestibility, and except for the essential amino acid methionine (Met), retention efficiency showed no statistics differences. IBE had the highest hydrolysis rate among the diets. Despite significant differences in diet, gilthead sea bream maintained a typically healthy liver morphology. However, fish fed TRO and INS diets showed shorter measurements in the distal intestine. In conclusion, the complete replacement of fish meal with organic materials, including rainbow trout byproducts, Iberian pork byproducts, and insect meal, offers several benefits in terms of digestibility, histology, and growth performance, promoting sustainable and healthy aquaculture practices.

The third study aimed to evaluate the feasibility of using various organic proteins in the diet of European sea bass. Juvenile sea bass with an average weight of 90 g were fed six diets twice daily with different compositions of organic proteins during a 125-day feeding trial. The control group (CON) received a diet with conventional fish meal as the main protein source. The other groups were fed diets incorporating organic byproducts of Iberian pork (IB), a combination of IB and insects (IB-IN), a mixture of IB and organic rainbow trout byproducts (IB-TR), a combination of organic rainbow trout byproducts and insects (TR-IN), and a mixed diet containing all these protein sources (MIX). The results indicated that the control diet produced the highest final weight and specific growth rate, followed by the TR-IN and IB-TR diets. The IB-TR diet exhibited the highest apparent digestibility coefficients (ADCs) for protein, while the TR-IN diet had the lowest. Histological analysis revealed that the control diet resulted in the largest core and hepatocyte diameters. The inclusion of insects (IN) in diets appeared to negatively affect performance, with fish fed diets containing IN showing slower growth and lower digestibility. Diets containing organic rainbow trout byproducts (TR) and organic Iberian pork byproducts (IB) showed growth rates close to the control, with acceptable digestibility. These findings suggest that certain sources of organic proteins can be effectively incorporated into the diets of juvenile sea bass, offering insights into sustainable and healthy aquaculture practices.

The fourth study tested 100% organic diets using mixtures of alternative protein sources, insect meal (IN), sea bass byproducts (SB), and Iberian pork byproducts (IB), for rainbow trout. The experimental diets consisted of 100% fishmeal replacement except the IN diet. The effects of these diets on growth, efficiency, productivity, and intestinal health were evaluated. Fish,

with an initial weight of 67.2 g, were fed twice a day for 150 days. The control diet with fish meal (FM) produced the highest final weight (298 g). The results indicated that the SB-FM, SB-IB, and SB-IN-IB diets had lower performance, while the FM-IN and IN-IB diets had the lowest final weight. Improvement in growth and nutrient utilization was observed in the SB-FM, SB-IB, and SB-IN-IB diets compared to the FM-IN and IN-IB diets. The lowest retention efficiencies were found in the IN-IB diet. The control and FM-IN diets had the highest apparent digestibility coefficients (ADCs) for protein, energy, calcium, and phosphorus. Enzymatic analysis revealed low levels of trypsin and chymotrypsin in the IN-IB and SB-IN-IB diets. Histological changes were observed in the liver and intestine in fish fed FM-IN and IN-IB diets. Microbiota analysis showed *Firmicutes, Spirochaetota*, and *Proteobacteria* as dominant phyla, with no significant differences between diets. Despite variations in growth, high replacement of fish meal did not significantly alter intestinal microbiota, possibly due to the dominance of *Firmicutes*. From an economic perspective, the SB-IB diets showed the lowest economic conversion ratio and the highest economic benefit index. In conclusion, fish meal replacement affected growth, with the best results in control containing FM and marine fish meal containing SB diets. However, animal byproducts offered the best economic outcomes.

These studies suggest the possibility of using organic ingredients, providing valuable insights to improve nutrition, growth, and sustainability in aquaculture. The findings contribute to promoting sustainable and organic seafood production to meet the growing demands of consumers while preserving the health of aquatic ecosystems. Despite promising results, further research and innovation are needed to fully realize the potential of organic feeding in aquaculture and reduce dependence on traditional fish meal in the industry.

Keywords: novel organic ingredients, organic by-products of animal origin, fishmeal replacement, sea bream, sea bass, rainbow trout, sustainable aquaculture

RESUM

L'acuicultura orgànica o ecològica està guanyant popularitat a mesura que els consumidors es tornen més conscients de les pràctiques de producció alimentària sostenibles i respectuoses amb el medi ambient. Les dietes amb ingredients orgànics són un component essencial de l'acuicultura ecològica. Aquestes dietes utilitzen ingredients orgànics i formulacions específiques per promoure una producció d'animals aquàtics més saludable i respectuosa amb el medi ambient. Es alineen amb els principis de l'agricultura orgànica, minimitzen l'ús de productes químics sintètics i busquen produir productes del mar més saludables i ambientalment responsables.

Aquesta tesi de doctorat reuneix quatre estudis diferents amb l'objectiu d'avançar en la nostra comprensió de la integració de nous ingredients orgànics a les dietes d'acuicultura i els seus efectes en diverses espècies, centrant-se en la dorada (*Sparus aurata*), la llobina (*Dicentrarchus labrax*) i la truita arc de Sant Martí (*Oncorhynchus mykiss*). Aquests estudis exploren el creixement, la composició corporal, la utilització dels aliments, la digestibilitat, les respostes histològiques i la composició de la microbiota en incorporar aliments i ingredients orgànics innovadors.

En el primer estudi, es va investigar l'efecte de l'aliment eco-orgànic en el creixement, l'eficiència nutricional, la utilització dels aliments i la composició corporal de la dorada. Es van provar sis dietes, incloent una dieta de control (CONT) sense ingredients orgànics, quatre dietes amb 100% d'ingredients orgànics (subproducte de truita - TRO, subproducte de llobina - SBS, subproductes avícoles - POU i barreja - MIX), i una dieta de control orgànica (ORG) amb ingredients orgànics i 30% de farina de peix. En l'experiment, de 70 dies de durada, es va alimentar els peixos dues vegades al dia, començant amb un pes inicial de 60,5 g. Els resultats van revelar que les taxes de creixement més altes es van observar en peixos alimentats amb les dietes ORG i CONT que contenien farina de peix. Per contra, la dieta POU va mostrar la taxa de creixement més baixa, la taxa de supervivència més baixa i el major índex de conversió alimentària (FCR). L'eficiència dels aminoàcids essencials va ser generalment alta en peixos alimentats amb les dietes ORG i CONT, amb diferències notables en l'eficiència de retenció d'àcids grassos en totes les dietes. La dieta CONT va mostrar la major eficiència de retenció, seguida per la dieta ORG. No obstant això, la taxa de conversió econòmica va ser menor per a CONT, SBS, TRO i MIX. En resum, l'ús de dietes orgàniques d'origen animal va afectar el rendiment de creixement de la Dorada, amb un potencial prometedor malgrat les variacions en les taxes de conversió econòmica entre diferents dietes.

En el segon estudi, es va investigar durant 120 dies la utilització de nous

ingredients orgànics per alimentar la dorada amb un pes inicial d'aproximadament 93 ± 3.82 g, centrant-se en el creixement, els paràmetres nutricionals, la digestibilitat i la histologia. Es van provar quatre dietes: una dieta de control orgànica (CON) i tres dietes amb 100% de materials orgànics - subproducte de truita arc de Sant Martí (TRO), subproducte de porc ibèric (IBE) i farina d'insectes com a font de proteïnes (INS). Al final de l'experiment, els peixos alimentats amb la dieta CON van mostrar el major pes, sense diferència significativa entre les dietes experimentals. El contingut de proteïnes brutes va ser major en els peixos alimentats amb les dietes TRO e INS, mentre que el major valor de greix brut es va observar en la dieta CON. Els peixos alimentats amb les dietes IBE e INS van demostrar una alta digestibilitat, i excepte per a l'aminoàcid essencial metionina (Met), l'eficiència de retenció no va mostrar diferències estadístiques. IBE va tenir la major taxa d'hidròlisi entre les dietes. Malgrat les diferències significatives a la dieta, la dorada va mantenir una morfologia hepàtica típicament saludable. No obstant això, els peixos alimentats amb les dietes TRO e INS van mostrar mesures més curtes a l'intestí distal. En conclusió, la substitució completa de la farina de peix amb materials orgànics, incloent-hi subproductes de truita arc de Sant Martí, de porc ibèric i farina d'insectes, ofereix diversos beneficis en termes de digestibilitat, histologia i rendiment de creixement, promocionant pràctiques acuícoles sostenibles i saludables.

El tercer estudi va tenir com a objectiu avaluar la viabilitat d'utilitzar diverses proteïnes orgàniques en la dieta de la llobina (Dicentrarchus labrax). Juvenils de llobina amb un pes mitjà de 90 ± 3.82 g van ser alimentats dues vegades al dia amb sis dietes amb diferents composicions de proteïnes orgàniques durant un assaig d'alimentació de 125 dies. El grup de control (CON) va rebre una dieta amb farina de peix convencional com a principal font de proteïnes. Els altres grups van ser alimentats amb dietes que incorporaven subproductes orgànics de porc ibèric (IB), una combinació de IB i insectes (IB-IN), una barreja de IB i subproductes orgànics de truita arc de Sant Martí (IB-TR), una combinació de subproductes orgànics de truita arc de Sant Martí i insectes (TR-IN) i una dieta mixta que contenia totes aquestes fonts de proteïnes (MIX). Els resultats van indicar que la dieta de control va produir el major pes final i la taxa de creixement específic, seguida per les dietes TR-IN e IB-TR. La dieta IB-TR va exhibir els coeficients de digestibilitat aparent (ADCs) més alts per a la proteïna, mentre que la dieta TR-IN va tenir els més baixos. L'anàlisi histològic va revelar que la dieta de control va donar lloc als diàmetres de nucli i hepatòcit més grans. La inclusió d'insectes (IN) a les dietes va semblar afectar negativament al rendiment, amb peixos alimentats amb dietes que contenien IN mostrant un creixement més lent i una menor digestibilitat. Les dietes que contenien subproductes orgànics de truita arc de Sant Martí (TR) i subproductes orgànics de porc ibèric (IB) van mostrar taxes de creixement properes a les del control, amb una digestibilitat acceptable. Aquests resultats suggereixen que certes fonts de proteïnes orgàniques es poden incorporar de manera efectiva a les dietes de llobina jove, oferint perspectives sobre pràctiques acuícoles sostenibles i saludables.

El quart estudi va assajar dietes 100% orgàniques utilitzant barreges de fonts alternatives de proteïnes, farina d'insectes (IN), subproductes de la llobina (SB) i subproductes del porc ibèric (IB), per a la truita arc de Sant Martí (Oncorhynchus mykiss). Les dietes experimentals consistien en un 100% de la substitució de la farina de peix excepte la dita IN. Es van avaluar els efectes d'aquestes dietes en el creixement, l'eficiència, la productivitat i la salut intestinal. Els peixos, amb un pes inicial de 67,2 g, van ser alimentats dues vegades al dia durant 150 dies. La dieta de control amb farina de peix (FM) va produir el major pes final (298 g). Els resultats van indicar que les dietes SB-FM, SB-IB e SB-IN-IB van tenir un rendiment menor, mentre que les dietes FM-IN e IN-IB van tenir el menor pes final. Es va observar una millora en el creixement i la utilització de nutrients en les dietes SB-FM, SB-IB i SB-IN-IB en comparació amb les dietes FM-IN e IN-IB. Les eficiències de retenció més baixes es van trobar en la dieta IN-IB. Les dietes control i FM-IN van tenir els coeficients de digestibilitat aparent (ADCs) més alts per a proteïnes, energia, calci i fòsfor. L'anàlisi enzimàtic va revelar baixos nivells de tripsina i quimotripsina en les dietes IN-IB i SB-IN-IB. Es van observar canvis histològics en el fetge i intestí en els peixos alimentats amb dietes FM-IN e IN-IB. L'anàlisi del microbiota va mostrar *Firmicutes, Spirochaetota i Proteobacteria* com a filos dominants, sense diferències significatives entre les dietes. Malgrat les variacions en el creixement, la substitució elevada de farina de peix no va alterar significativament el microbiota intestinal, possiblement degut a la dominància de *Firmicutes*. Des d'un punt de vista econòmic, les dietes SB-IB van mostrar el índex de conversió econòmica més baix i el índex de benefici econòmic més alt. En conclusió, la substitució de farina de peix va afectar el creixement, amb els millors resultats en les dietes de control que conté FM i de farina de peix d'origen marí que conté SB. No obstant això, els subproductes animals van oferir els millors resultats econòmics.

Aquests estudis suggereixen la possibilitat d'utilitzar ingredients eco-orgànics, proporcionant valuosos coneixements per millorar la nutrició, el creixement i la sostenibilitat de l'aquacultura. Les troballes contribueixen a promoure la producció sostenible i orgànica de productes del mar per satisfer les creixents demandes dels consumidors, alhora que preserven la salut dels ecosistemes aquàtics. Malgrat els resultats prometedors, es necessita més recerca i innovació per a realitzar plenament el potencial de l'alimentació

eco-orgànica en l'aquacultura i reduir la dependència de la indústria de la farina de peix tradicional.

Paraules clau: ingredients orgànics innovadors, subproductes orgànics d'origen animal, substitució de farina de peix, dorada, llobarro, truita arc de Sant Martí, aqüicultura sostenible.

RESUMEN

La acuicultura orgánica o ecológica está ganando popularidad a medida que los consumidores se vuelven más conscientes de las prácticas de producción alimentaria sostenibles y respetuosas con el medio ambiente. Las dietas con ingredientes orgánicos son un componente esencial de la acuicultura ecológica. Estas dietas utilizan ingredientes orgánicos y formulaciones específicas para promover una producción de animales acuáticos más saludable y respetuosa con el medio ambiente. Se alinean con los principios de la agricultura orgánica, minimizan el uso de productos químicos sintéticos y buscan producir productos del mar más saludables y ambientalmente responsables.

La presente tesis de doctorado reúne cuatro estudios distintos con el objetivo de avanzar en nuestra comprensión de la integración de nuevos ingredientes orgánicos en las dietas de acuicultura y sus efectos en diversas especies, centrándose en la dorada (*Sparus aurata*), la lubina (*Dicentrarchus labrax*) y la trucha arcoíris (*Oncorhynchus mykiss*). Estos estudios exploran el crecimiento, la composición corporal, la utilización del alimento, la digestibilidad, las respuestas histológicas y la composición de la microbiota al incorporar alimentos e ingredientes orgánicos novedosos.

En el primer estudio, se investigó el efecto del alimento eco-orgánico en el crecimiento, la eficiencia nutricional, la utilización del alimento y la composición corporal de la dorada. Se ensayaron seis dietas, incluyendo una dieta de control (CONT) sin ingredientes orgánicos, cuatro dietas con 100% de ingredientes orgánicos (subproducto de trucha - TRO, subproducto de lubina - SBS, subproductos avícolas - POU y mezcla - MIX), y una dieta de control orgánico (ORG) con ingredientes orgánicos y 30% de harina de pescado. En el experimento, de 70 días de duración, se alimentó a los peces dos veces al día, comenzando con un peso inicial de 60.5 g. Los resultados revelaron que las tasas de crecimiento más altas se observaron en peces alimentados con las dietas ORG y CONT que contenían harina de pescado. Por el contrario, la dieta POU mostró la tasa de crecimiento más baja, la tasa de supervivencia más baja y el mayor índice de conversión alimentaria (FCR). La eficiencia de aminoácidos esenciales fue generalmente alta en peces alimentados con las dietas ORG y CONT, con diferencias notables en la eficiencia de retención de ácidos grasos en todas las dietas. La dieta CONT mostró la mayor eficiencia de retención, seguida por la dieta ORG. Sin embargo, la tasa de conversión económica fue menor para CONT, SBS, TRO y MIX. En resumen, el uso

de dietas orgánicas de origen animal afectó el rendimiento de crecimiento de la Dorada, con un potencial prometedor a pesar de las variaciones en las tasas de conversión económica entre diferentes dietas.

En el segundo estudio, se investigó durante 120 días la utilización de nuevos ingredientes orgánicos para alimentar a la dorada de 93 ± 3.82 g de pesos inicial, centrándose en el crecimiento, los parámetros nutricionales, la digestibilidad y la histología. Se probaron cuatro dietas: una dieta de control orgánico (CON) y tres dietas con 100% de materiales orgánicos - subproducto de trucha arcoíris (TRO), subproducto de cerdo ibérico (IBE) y harina de insectos como fuente de proteínas (INS). Al final del experimento, los peces alimentados con la dieta CON mostraron el mayor peso, sin diferencia significativa entre las dietas experimentales. El contenido de proteínas crudas fue mayor en los peces alimentados con las dietas TRO e INS, mientras que el mayor valor de grasa cruda se observó en la dieta CON. Los peces alimentados con las dietas IBE e INS demostraron una alta digestibilidad, y excepto para el aminoácido esencial metionina (Met), la eficiencia de retención no mostró diferencias estadísticas. IBE tuvo la mayor tasa de hidrólisis entre las dietas. A pesar de las diferencias significativas en la dieta, la dorada mantuvo una morfología hepática típicamente saludable. Sin embargo, los peces alimentados con las dietas TRO e INS mostraron medidas más cortas en el intestino distal. En conclusión, la sustitución completa de harina de pescado con materiales orgánicos, incluyendo subproductos de trucha arcoíris, de cerdo ibérico y harina de insectos, ofrece varios beneficios en términos de digestibilidad, histología y rendimiento de crecimiento, promoviendo prácticas acuícolas sostenibles y saludables.

El tercer estudio tuvo como objetivo evaluar la viabilidad de utilizar diversas proteínas orgánicas en la dieta de la lubina (*Dicentrarchus labrax*). Juveniles de lubina con un peso promedio de 90 g fueron alimentadas dos veces al día con seis dietas con diferentes composiciones de proteínas orgánicas durante un ensayo de alimentación de 125 días. El grupo de control (CON) recibió una dieta con harina de pescado convencional como principal fuente de proteínas. Los otros grupos fueron alimentados con dietas que incorporaban subproductos orgánicos de cerdo ibérico (IB), una combinación de IB e insectos (IB-IN), una mezcla de IB y subproductos orgánicos de trucha arcoíris (IB-TR), una combinación de subproductos orgánicos de trucha arcoíris e insectos (TR-IN) y una dieta mixta que contenía todas estas fuentes de proteínas (MIX). Los resultados indicaron que la dieta de control produjo el mayor peso final y la tasa de crecimiento específico, seguida por las dietas TR-IN e IB-TR. La dieta IB-TR exhibió los coeficientes de digestibilidad aparente (ADCs) más altos para la proteína, mientras que la dieta TR-IN tuvo los más bajos. El análisis histológico reveló que la dieta de control resultó en los diámetros de núcleo y hepatocito más grandes. La inclusión de insectos (IN) en las dietas pareció afectar negativamente al rendimiento, con peces alimentados con dietas que contenían IN mostrando un crecimiento más lento y una menor digestibilidad. Las dietas que contenían subproductos orgánicos de trucha arcoíris (TR) y subproductos orgánicos de cerdo ibérico (IB) mostraron tasas de crecimiento cercanas a las del control, con una digestibilidad aceptable. Estos hallazgos sugieren que ciertas fuentes de proteínas orgánicas pueden incorporarse de manera efectiva en las dietas de lubina juvenil, ofreciendo perspectivas sobre prácticas acuícolas sostenibles y saludables.

El cuarto estudio ensayó dietas 100% orgánicas utilizando mezclas de fuentes alternativas de proteínas, harina de insectos (IN), subproductos de la lubina (SB) y subproductos del cerdo ibérico (IB), para la trucha arcoíris (*Oncorhynchus mykiss*). Las dietas experimentales consistían en un 100% de la sustitución de la harina de pescado excepto la dita IN. Se evaluaron los efectos de estas dietas en el crecimiento, la eficiencia, la productividad y la salud intestinal. Los peces, con un peso inicial de 67.2 g, fueron alimentados dos veces al día durante 150 días. La dieta de control con harina de pescado (FM) produjo el mayor peso final (298 g). Los resultados indicaron que las dietas SB-FM, SB-IB e SB-IN-IB tuvieron un rendimiento menor, mientras que las dietas FM-IN e IN-IB tuvieron el menor peso final. Se observó una mejora en el crecimiento y la utilización de nutrientes en las dietas SB-FM, SB-IB y SB-IN-IB en comparación con las dietas FM-IN e IN-IB. Las eficiencias de retención más bajas se encontraron en la dieta IN-IB. Las dietas control y FM-IN tuvieron los coeficientes de digestibilidad aparente (ADCs) más altos para proteínas, energía, calcio y fósforo. El análisis enzimático reveló bajos niveles de tripsina y quimotripsina en las dietas IN-IB y SB-IN-IB. Se observaron cambios histológicos en el hígado e intestino en los peces alimentados con dietas FM-IN e IN-IB. El análisis del microbiota mostró *Firmicutes, Spirochaetota y Proteobacteria* como filos dominantes, sin diferencias significativas entre las dietas. A pesar de las variaciones en el crecimiento, la sustitución elevada de harina de pescado no alteró significativamente el microbiota intestinal, posiblemente debido a la dominancia de *Firmicutes*. Desde el punto de vista económico, las dietas SB-IB mostraron el índice de conversión económica más bajo y el índice de beneficio económico más alto. En conclusión, la sustitución de harina de pescado afectó el crecimiento, con los mejores resultados en las dietas de control que contiene FM y de harina de pescado de origen marino

que contiene SB. Sin embargo, los subproductos animales ofrecieron los mejores resultados económicos.

Estos estudios sugieren la posibilidad de utilizar ingredientes eco-orgánicos, proporcionando valiosas ideas para mejorar la nutrición, el crecimiento y la sostenibilidad de la acuicultura. Los hallazgos contribuyen a promover la producción sostenible y orgánica de productos del mar para satisfacer las crecientes demandas de los consumidores, al tiempo que preservan la salud de los ecosistemas acuáticos. A pesar de los resultados prometedores, se necesita más investigación e innovación para realizar plenamente el potencial de la alimentación eco-orgánica en la acuicultura y reducir la dependencia de la industria de la harina de pescado tradicional.

Palabras claves: ingredientes orgánicos novedosos, subproductos orgánicos de origen animal, sustitución de harina de pescado, dorada, lubina, trucha arcoíris, acuicultura sostenible.

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LIST OF ABBREVIATIONS

AA: Amino acid
ABPs: animal byproducts
ADCs: apparent digestibility coefficients
ANF: Anti-nutritional factors
ANOVA: analysis of variance
AOAC: Association of Official Analytical Chemists
AQC: 6 aminoquinolylNhydroxysuccinimidyl carbamate
ARA: Arachidonic acid
CF: Condition factor
CI: Carcass index
CL: Crude lipid
CON: Control diet
CON: Organic control diet
CONT: Control diet
CP: Crude protein
CPM: Chlorpyrifos-methyl
DHA: Docosahexaenoic acid
DI: distal intestine
DM: Dry matter
E. Seabass: European seabass
E:S: enzyme/substrate ratio
EAA: Essential amino acids
ECR: Economic conversion ratio
EFSA: European Food Safety Authority
EPA: Eicosapentaenoic acid
EU: European Union
EUMOFA: European Market Observatory for Fisheries and Aquaculture
FAMEs: Total lipid fatty acid methyl esters
FAO: Food and Agriculture Organization
FCR: Feed conversion ratios
FEAP: Federation of European Aquaculture Producers
FI: Feed intake
FM: fish meal
FO: fish oil
FPH: Fish protein hydrolysate
FPH: Fish protein hydrolysates
FW: final weight
GHG: Greenhouse gas

GI: Gonadal index
GMOs: Genetically modified organisms
HI: Hermetia illucens
HIS: Hepatosomatic index
HPLC: High-performance liquid chromatography
IAA: Indispensable amino acid
IB: Iberian pig meal byproductsdiet
IBE: Organic visceral Iberian pig diet
IB-IN: Iberian pig meal byproductsand insect meal diet
IB-TR: Iberian pig and organic rainbow trout byproducts meal diet
INS: Insect diet
LA: Linoleic acid
LNA: Linolenic acid
LP: Lamina propria
MAPA: Ministerio de Agricultura, Pesca y Alimentación
MBM: Meat and bone meal
Met: Methionine
MI: Meat index
MIX: Insect meal, rainbow trout meal and Iberian pig byproducts meal diet
MIX: Organic trout, organic seabass, and poultry diet
ML: Muscle layer
MS: Member states
MUFA: Monounsaturated fatty acids
NEAA: Non-essential amino acids
NSP: Non-starch polysaccharides
ORG: Control organic diet
P: Phosphorus
PAPs: Processed animal proteins
PBM: Poultry byproduct meal
PER: Protein efficiency ratio
PFV: Productive fat value
PI: Proximal intestine
POU: Poultry diet
PPS: Plant protein sources
PPV: Productive protein value
PUFA: Polyunsaturated fatty acids
SBS: Organic seabass diet
SEM: Standard error
SFA: Saturated fatty acids
SGR: Specific growth rate
SL: Serous layer
SML: Submucous layer

SR: Survival rate
TAPs: Transformed animal proteins
TM: Tenebrio molitor
TR-IN: Rainbow trout byproducts and insect meal diet
TRO: Organic rest of rainbow trout diet
TRO: Organic trout diet
UPV: Polytechnic University of Valencia
VFI: Visceral fat index
VL: Villus length
VSI: Viscerosomatic index
VSS: Voluntary Sustainability Standard
VT: Villus thickness
WG: Weight gain

General Introduction

1. Aquaculture and organic aquaculture production and advancements

1.1 The status of global fisheries and aquaculture

The global production of aquatic animals was estimated to be approximately 178 million tonnes in 2020, slightly lower than the record of 179 million tonnes in 2018. Global aquatic animal production's combined first sale value was estimated to be around USD 406 billion, of which 88 Mt (USD 265 billion) provided by aquaculture production (Table 1) (FAO, 2022). Out of the total aquatic animal production, over 157 million tonnes (89 percent) were intended for human consumption, approximately 20.2 kg per person annually, as estimated. The remaining 20 million tonnes were allocated for non-food purposes, primarily for producing fishmeal (FM) and fish oil (FO), which accounted for 16 million tonnes (81 percent) of this non-food usage (FAO, 2022) (Table 1). Moreover, aquaculture accounted for 56 percent of the available aquatic animal food production for human consumption in 2020.

Table 1. Global production and utilization of fisheries and aquaculture resources (million tonnes, live weight).

	1990	2000	2010	2018	2019	2020
Production						
Capture						
Inland	7.1	9.3	11.3	12.0	12.1	11.5
Marine	81.9	81.6	79.8	84.5	80.1	78.8
Total capture	88.9	90.9	91.0	96.5	92.2	90.3
Aquaculture						
Inland	12.6	25.6	44.7	51.6	53.3	54.4
Marine	9.2	17.9	26.8	30.9	31.9	33.1
Total aquaculture	21.8	43.4	71.5	82.5	85.2	87.5
Total world fisheries and aquaculture	110.7	134.3	162.6	178.9	177.4	177.8
Utilization						
Human consumption	81.6	109.3	143.2	156.8	158.1	157.4
Non-food uses	29.1	25.0	19.3	22.2	19.3	20.4
Population (billions)	5.7	6.5	7.3	7.6	7.7	7.8
Per capita apparent consumption (kg)	14.3	16.8	19.5	20.5	20.5	20.2

Adapted from FAO, 2022.

1.2 The production of aquaculture on a global scale and trends

Global aquaculture production continued to grow in 2020 despite the COVID-19 pandemic's global impact, with variations observed across regions and individual countries. The total aquaculture production included 87.5 million tonnes of aquatic animals primarily used for human consumption, 35.1 million tonnes of algae for both food and non-food purposes, and 700 tonnes of shells and pearls for ornamental use, reaching a total of 122.6 million tonnes in live weight. This represented an increase of 6.7 million tonnes from 2018. The estimated farm gate value in 2020 was USD 281.5 billion, indicating growth from previous years (FAO, 2022).

Worldwide aquaculture production of animal species grew by 2.7 percent in 2020 compared to 2019, which was the lowest rate in over four decades. Finfish farming remained the dominant component, accounting for approximately 66 percent of global aquaculture. In 2020, farmed finfish reached 57.5 million tonnes, with inland aquaculture contributing 49.1 million tonnes and mariculture in the sea and coastal areas contributing 8.3 million tonnes (Figure 1). Other farmed aquatic animal species included molluscs, crustaceans, aquatic invertebrates, and semi-aquatic species (FAO, 2022).

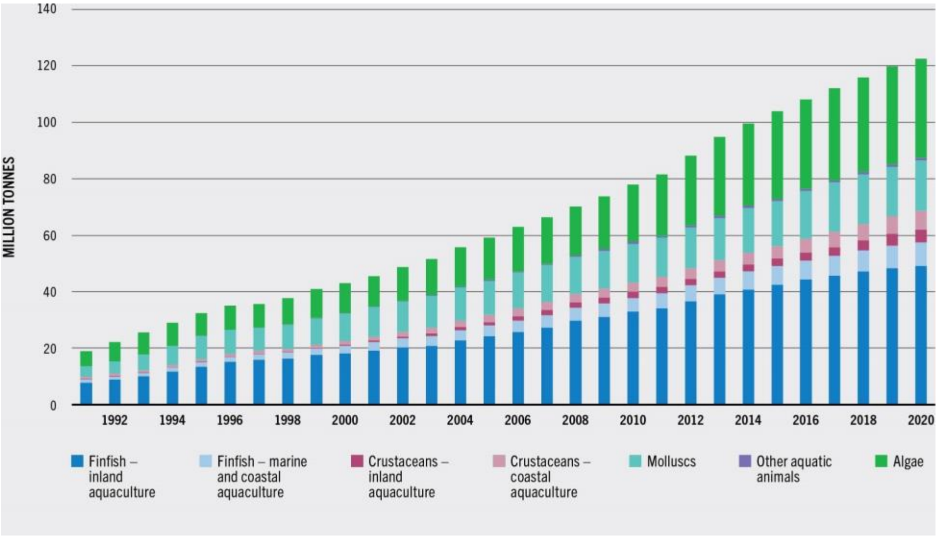


Figure 1. Global aquaculture production from 1991 to 2020. source FAO,2022

The production of algae, primarily seaweeds, increased by half a million tonnes in 2020, with China and Japan experiencing growth, while seaweed harvests decreased in Southeast Asia and Korea. At the regional level, African aquaculture (excluding algae) contracted slightly in output, mainly due to decreased production in Egypt and Nigeria. Other regions experienced growth, with Chile, China, and Norway leading in the Americas, Asia, and Europe (FAO, 2022).

Over the period 1990-2020, global aquaculture expanded by 609 percent, with an average growth rate of 6.7 percent per year. The growth rate gradually decreased from 9.5 percent (1990-2000) to 3.3 percent (2015-2020). Growth patterns in aquaculture development varied among regions, with Asia experiencing relatively steady growth in major aquaculture countries, while other regions had fluctuating growth, including negative growth in some years (FAO, 2022).

1.3 The position of the European Union (EU) in global aquaculture

The European Union (EU) holds a significant position in world aquaculture. In 2020, global aquaculture production reached 87 million tonnes, with China being the largest producer, accounting for 57% of the total production (49.6 million tonnes). India ranked second with 8.6 million tonnes, representing 10% of world production. The EU-27 secured the 10th position, contributing 1.3% of world production with 1.1 million tonnes produced (EUMOFA, 2022). In 2021, the European Union (EU) saw a total production of 1,142.5 thousand tons of aquaculture products. This figure reflects a notable increase of 3.6% compared to the quantity marketed in 2020, which amounted to 1,103 thousand tons (APROMAR, 2023). The list of producing countries within the European Union is led by Spain, which recorded a production of 271,060 tons in 2021, representing a decrease of -0.2% compared to 2020 (276,627 tons). France follows as the second-largest producer, with 198,886 tons in 2021, marking a 4.1% increase from 2020 (191,050 tons). Italy ranks third with 145,862 tons, showing a significant increase of 16.0% compared to 2020, while Greece recorded 143,926 tons, reflecting a 9.3% increase. Poland, with 44,787 tons, experienced a decrease of -6.1% compared to the previous year.
Spain accounted for 23.7% of the total production volume of the EU, followed by France at 17.4%, Italy at 12.8%, Greece at 12.6%, and Poland at 3.9%, emerging as the main producers within the region (APROMAR, 2023).

In terms of volume, the main species farmed in the EU in 2021 were mussels (423, 379 tonnes), trout (193,266 tonnes), gilthead seabream (103,130 tonnes), oysters (98,826 tonnes), European seabass (96,647 tonnes), carp (68,036 tonnes), bluefin tuna (26,320 tonnes), clams (25,232 tonnes), and salmon (14,512 tonnes). In terms of value, the top species farmed in the EU in 2021 were trout (EUR 665,5 million), European seabass (EUR 554,5 million), gilthead seabream (EUR 537,1 million), mussels (EUR 427,7 million), oysters (EUR 412,6 million), and bluefin tuna (EUR 358,9 million) (APROMAR, 2023).

1.4 Overview of organic aquaculture

Naturland, a German organization, took the lead in certifying organic aquaculture products, starting in 1995 with carp certification in Germany. This marked the inception of the first Voluntary Sustainability Standard (VSS) covering aquaculture production (Potts *et al.*, 2016). In 2005, IFOAM (IFOAM, 2005) – Organics International finalized its aquaculture standard. As of 2019, the reported production volume of organic aquaculture reached nearly 690,000 metric tons. Asia, primarily China and Europe accounted for 81% and 15% of the production, respectively. China had the highest production volume (561,200 metric tons), with Ireland (over 27,000 metric tons) and Chile (over 26,000 metric tons) following suit (Willer *et al.*, 2021).

It is worth noting that certain countries with substantial aquaculture production, like Brazil and Indonesia, did not disclose data on organic aquaculture. Hence, it is reasonable to assume that the organic aquaculture production volume is even higher. Approximately two-thirds of the total production data was available for species breakdown. According to the available information, organic mussels were the most produced species (over 27,000 metric tons), trailed by salmon (16,400 metric tons), and sturgeon (almost 1,800 metric tons) (Willer *et al.*, 2021).

1.5 The status of organic aquaculture production in the EU-27

In 2020, organic aquaculture production in the EU-27 reached approximately 74,032 tonnes, marking a significant increase compared to 2015, when it was 46,341 tonnes. This accounted for 6.4% of the total EU aquaculture production. Ireland was the leading producer, contributing over half of the nation's aquaculture output, focusing on salmon and mussels. Countries like Italy, France, the Netherlands, Spain, Germany, and Denmark had organic aquaculture production ranging from 5,000 to 10,000 tonnes, primarily involving shellfish such as mussels and oysters and finfish like trout and sturgeon (EUMOFA, 2022).

Bulgaria, Hungary, and Greece had organic aquaculture production ranging between 1,000 and 3,000 tonnes, involving mussels, finfish, European seabass, and gilthead seabream. Romania, Slovenia, Lithuania, Poland, Croatia, Austria, and Belgium reported organic aquaculture production below 1,000 tonnes, with some exceptions in Czechia and Portugal, where projects are ongoing. Mussels were the primary species, with 41,936 tonnes of certified organic production in 2020, accounting for 10% of the EU mussel production. The main contributing countries were the Netherlands, Italy, Germany, Denmark, France, and Spain. Salmon was the second main species, produced solely in Ireland, while trout ranked third. Carp production experienced a decrease, and oyster production showed an increase (EUMOFA, 2022).

Other species, like European seabass and gilthead seabream, amounted to 2,750 tonnes, with Greece being the leading MS contributing 57% of the EU production. Italy, France, Spain, Germany, Denmark, and Bulgaria demonstrated substantial growth in organic aquaculture, while Ireland and Hungary reported declining production levels (Figure 2) (EUMOFA, 2022).

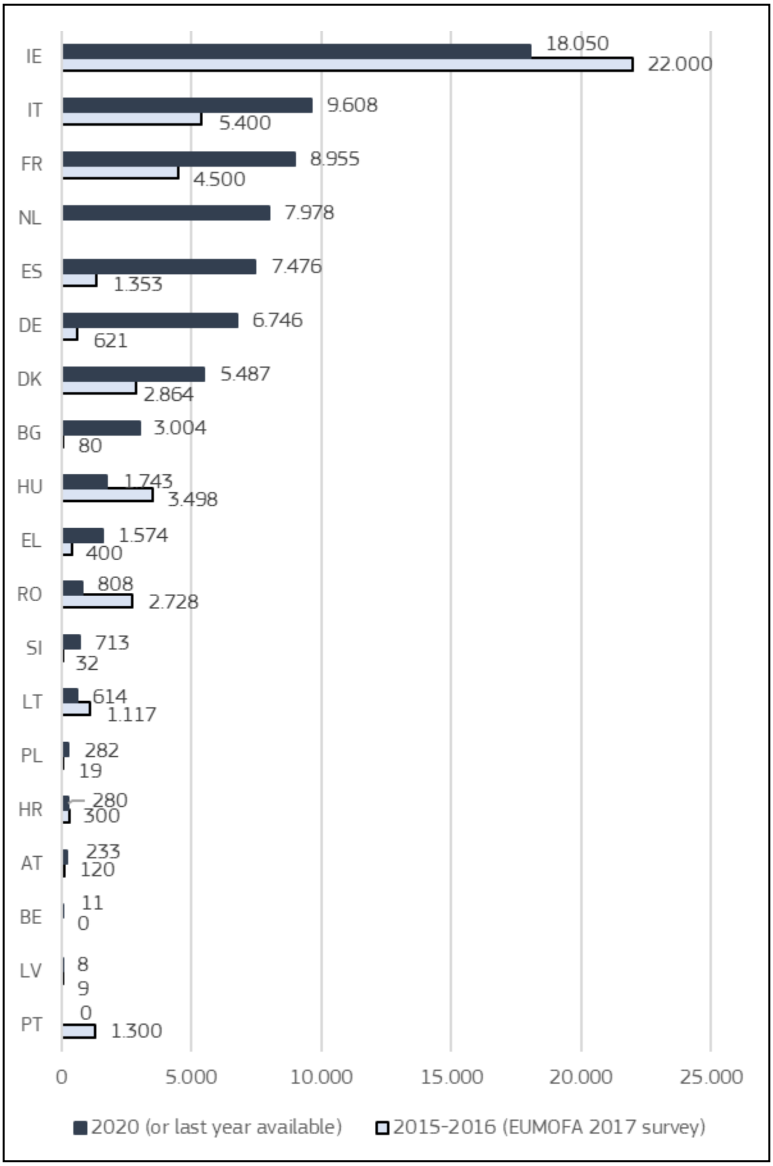


Figure 2. Contrasting the organic aquaculture volumes by Member States (MS) in 2020 (or the most recent available year) with the data from the EUMOFA survey in 2015-2016, presented in tonnes

Source: EUMOFA

1.6 The production of organic aquaculture in Spain

In 2020, organic aquaculture in Spain experienced remarkable growth, doubling its volume compared to 2015, reaching 7,476 tonnes. This accounted for 2.14% of the country's aquaculture production, with a sales value of EUR 80.25 million, representing 16% of the total sales value of Spanish aquaculture (MAPA, 2021). Galicia, Andalucía, and Castilla-La Mancha were the main regions contributing to organic aquaculture production, with Galicia hosting 140 mussel farms out of the total 174 aquaculture farms in Spain (MAPA, 2021).

Mussels were the primary species, comprising 42% of the total organic volume, with significant growth observed in 2018 due to increased production by Galician producers. Sturgeon ranked as the second species with 2,520 tonnes, maintaining stability between 2016 and 2019 and experiencing a 43% increase in 2020, primarily in Andalucía (MAPA, 2021). Trout accounted for 12% of the total organic volume, reaching 917 tonnes in 2020, displaying a volume 2.5 times higher than in 2015, mainly produced in Castilla-La Mancha and La Rioja (MAPA, 2021).

The production of algae witnessed substantial growth, soaring ten times higher in 2020 compared to 2015, totalling 564 tonnes, with Galicia and Asturias as the primary regions for algae production (MAPA, 2021). Organic European seabass and gilthead seabream production remained limited, with 210 tonnes and 124 tonnes, respectively, showing an increasing trend mainly in the Valencia Region (MAPA, 2021). Organic oyster production was minimal, amounting to 37 tonnes in 2020, mainly sourced from Asturias. According to MAPA (MAPA, 2021) data, Spanish organic seafood imports reached EUR 22 million in 2020, with exports reaching EUR 18 million, resulting in a trade balance of -EUR 4 million. The estimated consumption of organic seafood in 2020 was EUR 59 million, accounting for 0.59% of total seafood consumption (MAPA, 2021).

Organic aquaculture products constituted 2.3% of the total consumption of organic food in Spain in 2020, as reported by MAPA. This percentage is significantly lower than the share of aquaculture products in total food consumption (12.2%). Still, a comprehensive comparison may be influenced by including wild-caught products in total food consumption (EUMOFA, 2022).

1.7 Challenges and Prospects in Organic Aquaculture: A Mediterranean Perspective

Organic aquaculture is gaining momentum as a sustainable alternative to conventional fish farming. However, it faces various challenges that hinder its widespread adoption and success. The major challenges in organic aquaculture are difficulty in sourcing certified organic ingredients and the high cost of organic feed. These challenges stem from various factors, such as certification standards, which can be stringent and complex, making it challenging for producers to meet the requirements. Moreover, sourcing organic feed ingredients can be difficult due to limited availability and higher costs compared to their conventional counterparts. Disease management poses another challenge, as organic aquaculture practices often rely on natural methods rather than chemical interventions, requiring careful monitoring and management strategies to prevent disease outbreaks. Furthermore, organic aquaculture must address environmental impact concerns, ensure sustainable production methods, and minimize negative effects on surrounding ecosystems. Finally, meeting market demand for organic aquaculture products adds another layer of complexity, as consumer preferences and willingness to pay a premium for organic products fluctuate. Addressing these challenges is essential for successfully implementing and growing organic aquaculture practices. Organic aquaculture is recognized for its commitment to ecological balance, animal welfare, and reduced environmental impact (EC, 2021). Despite its potential benefits, organic aquaculture encounters a range of challenges that require immediate attention to ensure its long-term sustainability (Sethi *et al*., 2023).

One of the most significant challenges in organic aquaculture is sourcing sustainable and organic feed alternatives. Reducing reliance on conventional fishmeal and fish oil, which contribute to overfishing and depletion of marine resources, is crucial for ensuring the ecological balance of aquatic ecosystems (Rector *et al*., 2023). Aquaculture, while efficient in producing protein-rich and nutritious foods (Osmundsen *et al*., 2020)), often faces criticism for its unsustainable practices, particularly concerning the use of aquafeeds. (Cottrell *et al*., 2020; Osmundsen *et al*., 2020). These conventional feeds are often derived from wild-caught fish stocks, contributing to habitat destruction and biodiversity loss and its adverse environmental impacts (Valenti *et al*., 2018)). Moreover, the intensive use of conventional feeds can lead to eutrophication and pollution in aquaculture systems (Naylor *et al*., 2021) Transitioning to sustainable and organic feed alternatives is essential for mitigating these environmental impacts and ensuring the long-term viability of aquaculture practices. However, sourcing such alternatives presents challenges due to limited availability, higher costs, and the need for certification to meet organic standards. Addressing these challenges is critical for promoting the sustainability and resilience of organic aquaculture systems (Ahmed, 2020).

Aquafeeds typically include fish oil and fishmeal, obtained from small pelagic species like anchovies, herrings, and sardines, as well as fish byproducts such as discards and trimmings (FAO, 2022). Historically, fish oil and fishmeal were favoured for their high palatability and their supply of essential amino acids and fatty acids for most farmed fish species (Turchini *et al*., 2019). However, the global supply of fish oil and fishmeal is leveling off (Cottrell *et al*., 2020; Naylor *et al*., 2021), prompting the aquaculture sector to progressively reduce its reliance on these ingredients for sustainable growth (Cottrell *et al*., 2020). The development and implementation of rigorous organic certification standards specific to aquaculture systems are essential. These standards should encompass factors like feed composition, water quality, and waste management to guarantee organic integrity (Rector *et al*., 2023).

Organic aquaculture systems face increased vulnerability to disease outbreaks due to limited use of antibiotics and chemicals. Implementing effective disease prevention and management strategies is vital to safeguard the health and welfare of farmed fish in organic aquaculture systems. However, achieving this goal can be challenging due to the limited availability of approved organic treatments and preventive measures. Organic certification standards often restrict the use of synthetic chemicals and antibiotics, requiring farmers to rely on natural remedies, biosecurity measures, and other non-chemical interventions.

To achieve sustainable organic aquaculture, minimizing its environmental footprint is imperative. Organic aquaculture seeks to minimize environmental impacts by reducing chemical inputs, conserving natural resources, and promoting ecosystem health. However, organic systems may still face challenges related to nutrient pollution, habitat degradation, and the escape of farmed fish into the wild (Rector *et al*., 2023)..

Addressing these environmental issues requires a holistic approach that considers the entire production cycle, from feed production to waste management. The environmental impact of intensive fed aquaculture is primarily influenced by the feed used, as it plays a crucial role in determining the yields of the aquaculture system (Tacon, 2020). Therefore, sourcing sustainable and organic feed alternatives is essential for minimizing environmental impacts in organic aquaculture.

Feed ingredients' production, processing, and supply chain play a significant role in aquaculture's greenhouse gas (GHG) footprint (McKuin *et al.*, 2022). The production of feed ingredients, such as fishmeal and fish oil, often involves resource-intensive processes that contribute to emissions and environmental degradation. Therefore, transitioning to more sustainable feed sources and improving feed efficiency are essential steps towards reducing the environmental footprint of organic aquaculture systems (McKuin *et al.*, 2022).

Consumer awareness and demand for organic aquaculture products play a pivotal role in driving the industry's growth and economic viability. Creating effective marketing strategies and promoting the benefits of organic aquaculture can encourage consumer preference for sustainable seafood options (Rector *et al.*, 2023). Consumer awareness and demand for organic aquaculture products are indeed on the rise, driven by concerns about sustainability, health, and transparency. By positioning organic aquaculture as a viable and desirable option in the seafood market, producers can capitalize on this trend and contribute to the growth and economic viability of the organic aquaculture industry.

In order to tackle these challenges, it is essential to focus on exploring new feeding ingredients that are environmentally sustainable and well-suited for aquaculture nutrition as one option for making aquaculture more sustainable. Additionally, adopting ecosystem-based management practices will play a crucial role in ensuring the long-term sustainability of aquaculture production.

1.8 Organic aquaculture for more sustainability: Prominent species in Mediterranean aquaculture

The Mediterranean traditional and organic aquaculture sector predominantly focuses on three main fish species:

Gilthead Seabream (*Sparus aurata*): is a highly valued fish species in the Mediterranean region. It is appreciated for its delicate flavour and firm white flesh, making it a popular choice in both local and international markets.

European Seabass (*Dicentrarchus labrax*): is another prominent fish species farmed in Mediterranean aquaculture. It is prized for its mild taste and versatile culinary applications, making it a sought-after choice among consumers.

Rainbow Trout (*Oncorhynchus mykiss*): While seabream and seabass dominate marine aquaculture, rainbow trout holds significance in freshwater aquaculture activities in the Mediterranean region. It is valued for its rapid growth, adaptability to various farming systems, and appealing pink flesh.

These three species play a crucial role in meeting the demand for high-quality and sustainable seafood products in the Mediterranean aquaculture industry. Their successful production and market popularity contribute significantly to the region's aquaculture sector's growth and development.

Seabream and Seabass

Due to their similar environmental and biological requirements, seabream and seabass share similar forms of production, making it possible for them to be grown together on the same farms and even be replaced with each other interchangeably. The market dynamics of one species also influence the other, making it essential to conduct a joint analysis of their production.

The combined total production of seabream and seabass in Europe and the rest of the Mediterranean in 2022 reached 622,000 tonnes, as per consolidated data from APROMAR (2023).

Organic farming of European seabass and gilthead seabream is exclusively practiced along the Mediterranean coast, involving member states such as Italy, Greece, France, Croatia, and Spain. Within the European Union (EU) 27, the organic production of European seabass and gilthead seabream totalled 2,750 tonnes in 2020, accounting for 1.5% of the total production of both species. Over the span of five years (2015-2020), organic certification saw a notable increase, rising from just over 1,000 tonnes in 2015 to 2,750 tonnes in 2020, primarily driven by the growth in Greek production (EUMOFA, 2022).

Among the member states, Greece stands out with the highest organic production, reaching 1,574 tonnes in 2020, which constitutes 1.6% of the Greek production of both species. France recorded the highest proportion of organic production in the total output of European seabass and gilthead seabream in the same year, accounting for 5.2%, with Gloria Maris leading the way (other farms having limited organic production). Additionally, there is a noticeable upward trend in organic production in Spain, increasing from 116 tonnes in 2015 to 333 tonnes in 2020, and in Italy, rising from 144 tonnes to nearly 350 tonnes. Furthermore, Portugal has a unit engaged in experimental organic production of European seabass and gilthead seabream.

The seabream and seabass are widely produced in almost all Mediterranean countries. Hatcheries carefully control the production of eggs from breeding individuals. Each female can lay 250,000 eggs of 1 mm in diameter per kilo of weight. During their initial month of life in aquaculture, the larvae are nourished with live organisms such as rotifers and artemia. Subsequently, they are introduced to a diet comprising feed derived from natural raw materials. The predominant technique for producing seabream and seabass involves intensive systems, with the utilization of water cages. The seabass takes approximately 20 to 24 months to reach a weight of 400g from the time it hatches from the egg. Commercially, it is available in sizes ranging from 250 g to over 2,500 g (FAO Home, 2023**)**.

Gilthead seabream (*Sparus aurata, L.*)

The gilthead seabream (*Sparus aurata*) belongs to the class Osteichthyes, the order Perciformes, and the family *Sparidae*. The species exhibits a high oval body that is laterally flattened, accompanied by a large head featuring an arched profile. Its coloration is silvery gray, with a dark spot at the commencement of the lateral line and a small scarlet band on the upper edge of the operculum. A striking golden band can be observed between its eyes. The fish boasts a forked caudal fin and can grow up to 57 cm in length. As a proterandric hermaphrodite, it initially matures as a male and transitions to female during the second or third year. Its lifespan can exceed 10 years.

The most widely employed method of seabream production is through intensive systems, with water cages being a prominent approach. This system is utilized during the pre-growing and on-growing stages, following other phases such as reproduction, larval culture, and juvenile growing. Water-cage production offers several advantages over in-land methods, as it eliminates the need for water pumping, oxygenation, and treatment, simplifying operations and reducing costs. However, a drawback is the inability to regulate water temperature, resulting in a longer time for fish to reach the desired commercial weight.

According to data from APROMAR (2023), the total production of seabream in Europe and the rest of the Mediterranean was approximately 320,630 tonnes in 2022, showing a significant increase of 1.8% increase compared to 2021 (314,964 tonnes). The projected estimate for 2023 indicates a slight decrease to reach around 315,500 tonnes. The total value of Mediterranean seabream production in 2022, calculated at the first sale, amounts to 1,574.8 million euros. The production of seabream is carried out in 20 countries, with the primary producers being Turkey with 133,500 tonnes (41.6% of total production), Greece with 67,000 tonnes (22.7%), Egypt with 36,000 tonnes (11.2%), Tunisia with 16,000 tonnes (5.0%), and Spain with 8,932 tonnes (2.8%). Italy, Cyprus, and Croatia also contribute to its production, while minor productions are observed in other countries like Malta, France, Portugal, Albania, Algeria, United Arab Emirates, and Bosnia.

The production of juvenile seabream in Europe (including Turkey) in 2022 is estimated at 732.9 million units, representing an increase of 2.6% compared to 2021 (714.4 million units). Turkey is the main producing country with 240 million units, followed by Greece with 218 million units. Italy, France, and Spain follow, producing 130 million, 59.7 million, and 30.2 million juveniles, respectively. However, it is essential to acknowledge the difficulty in accurately contrasting these figures, especially in Greece and Turkey. The estimate for 2023 suggests a further increase in juvenile seabream production by 0.5%, reaching 733 million units APROMAR (2023).

In Spain, the production of seabream in 2022 was 8,932 tonnes, showing a decrease of 7.3% compared to the previous year with 9,632 tonnes. It is estimated to increase to 11,000 tonnes in 2023. The Valencian Community led the seabream production in Spain with 5,620 tonnes (63% of the total), followed by the Region of Murcia (1,327 tonnes, 15% of the total), Andalusia (815 tonnes, 9%), and the Canary Islands (790 tonnes, 9% of the total). Productions decreased in the Region of Murcia and Andalusia APROMAR (2023).

Overall, aquaculture accounts for 95.8% of the total supply of seabream worldwide, with only 4.2% coming from capture fisheries. In Spain, aquaculture is responsible for 92.8% of the seabream supply, according to FAO data (**Figure 3**).

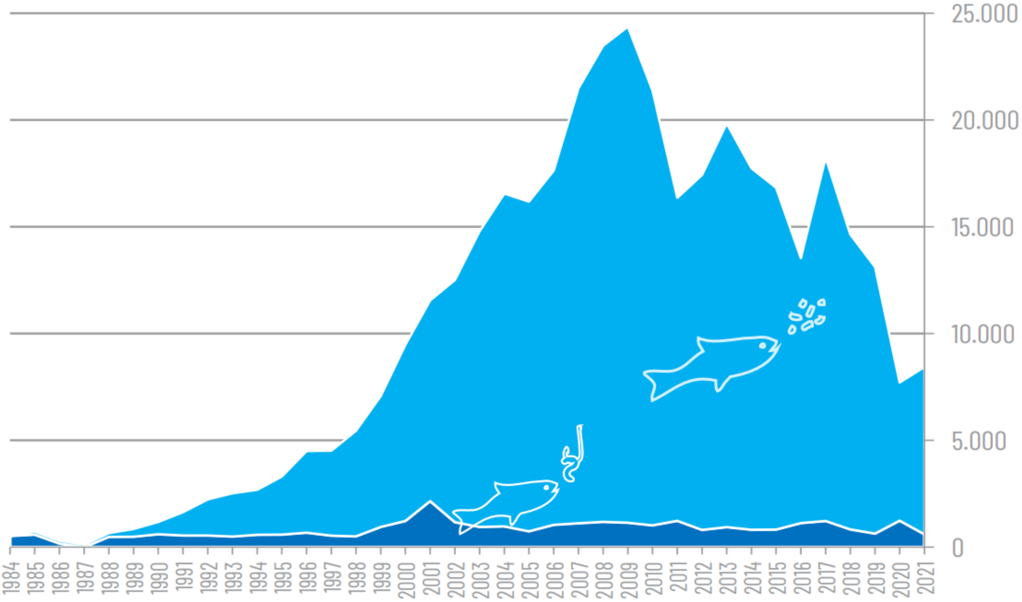


Figure 3. Historical trend in world production (in tonnes) of seabream (*Sparus aurata*) from 1984 to 2021, encompassing both aquaculture and capture fisheries, APROMAR 2023

Considerable research has been conducted on the nutritional needs of gilthead seabream during its growth phase, encompassing studies on dietary protein (45-46% in juveniles), amino acids (AA) requirements, dietary lipid levels (21-22%), and essential fatty acids (Benedito-Palos *et al*., 2009; Mongile *et al*., 2014; Peres & Oliva-Teles, 2009). While a carbohydrate level of up to 20% is beneficial, exceeding this level may negatively impact fish growth and feed efficiency (Enes *et al*., 2011).

Nonetheless, our understanding of the gilthead seabream's complete nutritional needs, particularly concerning vitamins and minerals, remains incomplete (Oliva-Teles, 2000) despite ongoing research efforts (Jobling, 2016).

European Seabass (*Dicentrarchus labrax, L.*)

The European seabass (*Dicentrarchus labrax*) belongs to the class Osteichthyes, the order Perciformes, and the family *Moronidae*. The seabass exhibits a streamlined and robust body covered with large scales. It possesses a pointed head with small nasal openings, small eyes, and a large mouth. The lower jaw is slightly prominent. Its coloration is leaden gray, darker on the dorsal part with silver sides, and a black spot on the operculum. The caudal fin is slightly forked. It can grow up to 70 cm in length and is known for tolerating temperature and water salinity variations. Sexual maturity is typically attained at 2-4 years, and its lifespan is estimated to be around 30 years (FAO Home, 2023**)**.

The European Seabass (*Dicentrarchus labrax L.*) is a significant marine finfish species in Europe, particularly in the Mediterranean, where it is widely produced in aquaculture. The total seabass production in Europe and the Mediterranean region in 2022 reached 301,420 tonnes, based on comprehensive data from APROMAR (2023). This represents a 1.2% increase compared to 2021 (297,742 tonnes). It is projected that the production remains stable or slightly higher in 2023 (0.5% increase). The total value of first-sale production for seabass in 2022 was approximately 1,488.7 million euros. The major seabass producing countries include Turkey, contributing 156,000 tonnes (51.8% of the total), Greece with 54,000 tonnes (17.9%), Egypt with 35,000 tonnes (11.6%), and Spain with 23,600 tonnes (8.9%). Additionally, seabass is also produced in Italy, Croatia, France, Tunisia, Portugal, Cyprus, the United Kingdom, Bosnia, Algeria, Montenegro, and Morocco. Turkey was the main producing country with 230 million units, followed by Greece with 164 million and Spain with 60 million. France produced approximately 53.2 million units and Italy 50 million. A slightly lower production is estimated for 2023, around 567 million European seabass juveniles (APROMAR, 2023).

Although some seabass is still caught through capture fishing in various Mediterranean Sea and Atlantic Ocean countries, the total capture was 5,570 tonnes in 2021 (a 15.2% increase compared to the previous year), as reported by FAO. Nonetheless, the majority, 98.2%, is sourced from aquaculture (**Figure 4**). In Spain, the seabass production reflecting a decrease of 1.3% compared to 2021. The leading regions in production were the Region of Murcia (7,244 tonnes, 31% of the total), followed by Andalusia with 6,020 tonnes (25% of the total), the Valencian Community (5,240 tonnes, 22% of the total), the Canary Islands (4,948 tonnes, 21%), and Catalonia with 170 tonnes (1% of the total). A growth of 1.2% is forecasted for 2023 with a European seabass harvest in Spain of 23,910 tonnes (APROMAR, 2023).

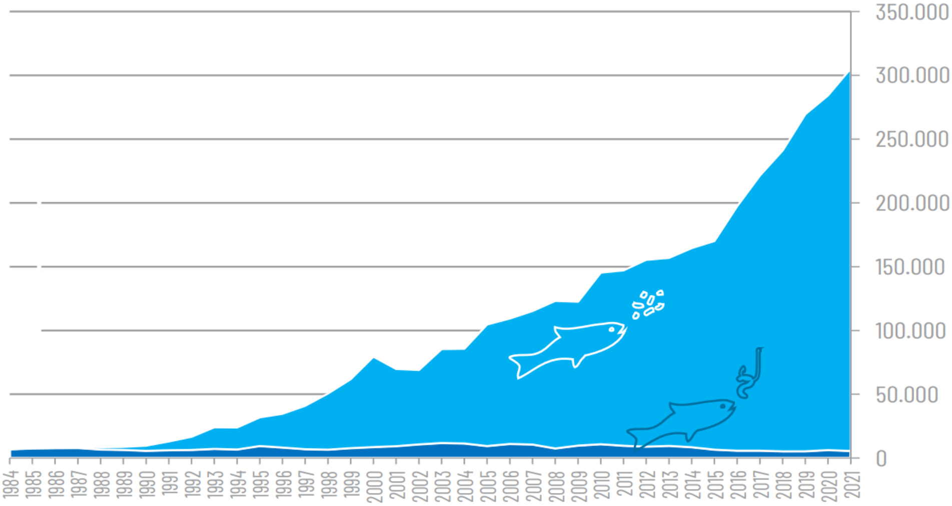


Figure 4. Historical trend in world production (in tonnes) of seabass (*Dicentrarchus labrax*) from 1984 to 2020, encompassing both aquaculture and capture fisheries, APROMAR 2023.

Considerable research has been conducted on the nutritional needs of E. Seabass during its growth phase. Regarding the indispensable amino acid (IAA) needs of E. Seabass, there are existing quantitative estimates for sulphur amino acids, lysine, tryptophan, and arginine (lanari *et al*., 1993; Thebault *et al*., 1985; Tibaldi *et al*., 1994; Tibaldi & Lanari, 1991). Through a dose-response study and the use of semi-purified diets, the requirement levels for methionine and total sulphur amino acids were brought down from approximately 40 g/kg dietary protein to 18-20 g/kg dietary protein (Tulli *et al*., 2010).

Research has explored the nutritional requirements of seabass throughout its growth phases, recommending dietary levels for protein (45% to 55%), lipids (6% to 18%, with an emphasis on essential fatty acids), and carbohydrates (up to 20%) (Ambasankar *et al*., 2009)**.** Concerning protein requirements, juvenile seabass has an absolute protein requirement of 4-5g/kg body weight/day for fish weighing around 100-200 g, which is comparatively lower than values for other faster-growing teleosts (Dias *et al*., 2003; Kaushik *et al*., 1995; Tacon & Cowey, 1985). For essential fatty acids (EFA), juvenile seabass has a reported quantitative requirement of 10 g/kg diet. Additionally, the phospholipids requirement is 120 g/kg of total lipids in larvae and 2-3 g/kg of total lipids in juveniles (Cahu *et al*., 2003).

Seabass can absorb and assimilate dietary starch, allowing for the storage of liver glycogen. However, this rate decreases with higher dietary starch levels and reduced water temperature (Enes *et al*., 2006; Moreira *et al*., 2008).

Rainbow trout (*Oncorhynchus mykiss, Walbaum*)

Rainbow Trout (*Oncorhynchus mykiss*) is a remarkable species belonging to Actinopterygii Order: Salmoniformes and Family: *Salmonidae*. It is easily recognizable by its elongated, fusiform body shape and the presence of an adipose fin. The coloration of this magnificent fish varies from blue to olive green, with a stunning pink iridescent band along the lateral line and a silver hue below it. Notably, its sides, head, and fins are adorned with tiny black dots, adding to its unique beauty. The intensity ranges from deep dark shades to bright silver, making it a sight.

Rainbow Trout is widely produced in many parts of the world. Females can produce up to 2,000 eggs per kg of body weight, which are relatively large, measuring between 3 to 7 mm in diameter. After hatching, the fry initially relies on the reserve food provided by the vitelline vesicle for sustenance. As they grow, they transition to a diet based on feed made from natural ingredients. Aquaculture farms employ various methods, such as ponds on land, concrete or fiber facilities, and even cages in fresh and saltwater environments. Typically, it takes about ten months from hatching for rainbow trout to reach the desired ration size, around 250-300 g. However, commercially grown rainbow trout can reach several kilograms in weight (FAO Home, 2023).

Global aquaculture production of rainbow trout (Oncorhynchus mykiss) in 2021 amounted to 948,663 tonnes, representing a decrease of -0.4% compared to the previous year's production of 952,342 tonnes. The leading producing countries were Iran with 193,852 tonnes (20.4% of the global total), Turkey with 165,683 tonnes (17.5%), Norway with 94,660 tonnes (10%), Chile with 56,656 tonnes (6.0%), the Russian Federation with 52,929 tonnes (5.6%), and Peru with 51,571 tonnes (5.4%). Other significant producers by volume include China, Russia, Italy, Denmark, France, Colombia, and the USA. Rainbow trout is cultivated in 79 countries across all five continents, despite its North American origin (APROMAR, 2023).

The majority of rainbow trout production occurs in freshwater (70%), but a significant portion of its farming is completed in saltwater, especially in

Chile and Norway. Commercial capture fisheries of rainbow trout are minimal, accounting for only 1,442 tonnes worldwide in 2021, in countries such as Uzbekistan, Finland, Mexico, Peru, and the United Kingdom. Rainbow trout production in Spain in 2022 is estimated at 16,328 tonnes, a 4.1% increase compared to the previous year. A slight decrease is projected for 2023, with around 15,500 tonnes, although both productions are far from the peak of 35,384 tonnes in 2001. The main producing regions include Castilla y León, Galicia, Andalusia, Catalonia, La Rioja, Castilla-La Mancha, Asturias, and Aragon (APROMAR, 2023).

Organic trout production is widespread across EU member states, with the total volume reaching 4,590 tonnes in 2020, equivalent to approximately 2% of the total farmed trout production in the EU for that year. France emerged as the leading producer in 2020, accounting for 52% of the total volume, followed by Spain (19%), Denmark (11%), Italy (9%), and Germany (7%).

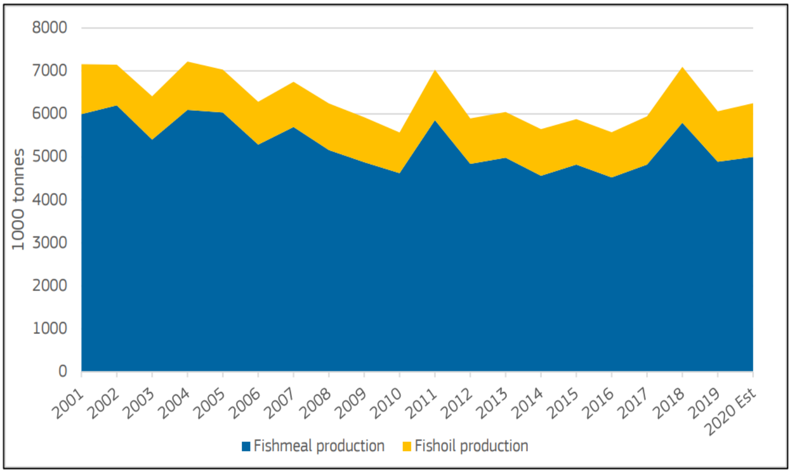
Since 2015, organic farmed trout production in the EU has experienced a decline, primarily attributed to significant decreases in Denmark, and to a lesser extent in France and Italy. Stakeholders interviewed attribute this decline in rainbow trout production to a discrepancy between EU organic legislation and Danish regulations governing fish farming (EUMOFA, 2022).

Like many other animal and fish species in rainbow trout farming, feed cost represents a significant expense, accounting for approximately 40-70% of the total costs (Lasner *et al*., 2017). Therefore, the preparation, nutritional value, and feeding strategy of the feed are crucial factors that directly impact the profitability of a rainbow trout farm (Kamalam *et al*., 2019). In 2012, the total feed consumption in trout farming reached around 1.1 million tonnes (Kamalam *et al*., 2019), and the feed efficiency ranged from 0.8 to 1.1, depending on the farming system and country (Lasner *et al*., 2017; Tacon & Metian, 2015).

In the 1990s, rainbow trout were primarily fed moist feeds composed mainly of protein-rich animal byproducts. Subsequently, semi-moist and dry feeds were developed to provide all the essential macronutrients, fatty acids, amino acids, and vitamins (Hardy, 2003). Presently, nutrient-dense extruded feeds are formulated to meet the specific requirements of rainbow trout, with digestible energy at 17.6 kJ/g, protein content ranging from 40% to 50%, and lipids at 16-24% (Hardy, 2003; Jobling, 2012). These formulations may vary based on fish size, farming environment, systems, and market preferences (Kamalam *et al*., 2019).

2. Moving towards sustainable production: the substitution of fishmeal

Fish oil and fishmeal are crucial in aquafeeds, providing essential lipids, particularly n-3 PUFA, and proteins for fish nutrition (Ido & Kaneta, 2020; Macusi *et al*., 2023; Olsen & Hasan, 2012; Turchini *et al*., 2009). These valuable ingredients are derived from whole fish, fish trimmings, and other fish processing byproducts. While small aquatic species, such as anchoveta (*Engraulis ringens*), are commonly used for fishmeal and fish oil production due to their high oil yields, they are rarely utilized for direct human consumption. The reliance on high levels of fishmeal and fish oil in aquafeeds has raised concerns about the potential impact on fish populations and the sustainability of the aquaculture sector (Hardy, 2010). Over the last two decades, there has been a decline in the proportion of world fisheries allocated to fishmeal and fish oil production. Between 2001 and 2010, the annual average fishmeal production exceeded 5.5 million tonnes; from 2011 to 2020, it remained around 5 million tonnes. In 2018, global fishmeal production peaked at 5.8 million tonnes, a 20% increase from the previous year. Similarly, fish oil production ranged between 0.8 and 1.3 million tonnes per year. Fish oil production reached its highest level in the past 20 years, nearing 1.3 million tonnes (Figure 5). This boost in production was mainly attributed to substantial catches of Peruvian anchoveta. However, the trend changed in 2019 and 2020, with estimated global fishmeal production dropping to 4.9 and 5 million tonnes, respectively, and fish oil production declining to 1.17 and 1.25 million tonnes. This decrease was mainly due to reduced catches in Peru, impacting the overall production figures (EUMOFA, 2021).

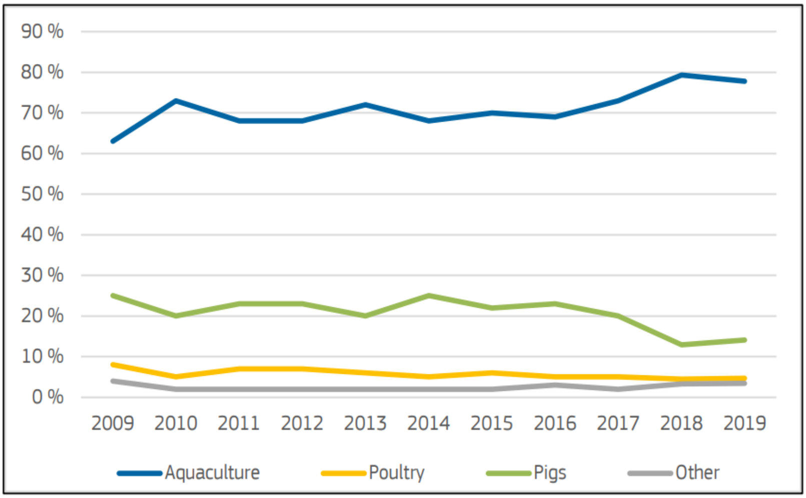


Source: IFFO

Figure 5. The global production trends of fishmeal and fish oil (in tonnes) from 2001 to 2020.

The soaring demand for aquaculture products led to a surge in aquafeed production. Consequently, the limited availability of fishmeal and fish oil, coupled with their increasing prices, prompted researchers and the industry to explore alternative sources (Tacon and Metian, 2008). By 2006, the cost of fishmeal rose significantly, compelling a shift in aquafeed formulation to reduce reliance on these ingredients (Hardy, 2010). The necessary percentages of fishmeal in feeds have been halved for carnivorous species, resulting in commercial feeds containing approximately 15% fishmeal. Over the past decade, there have been no significant shifts in the global utilization of fishmeal across various sectors. However, in recent years, aquaculture has seen a rising share of fishmeal usage. In 2009, aquaculture accounted for 63% of fishmeal consumption, which remained relatively stable at around 70% from 2010 to 2017-2019 before increasing to 78%. In 2019, crustaceans consumed approximately 25% of fishmeal in aquaculture, followed by salmon and trout at 15%, marine fish at 17%, and freshwater species at 21%. The remaining fishmeal was distributed among tilapias, cyprinids, and eels. The Asian region, particularly China and other Asiatic countries, dominated fishmeal consumption in aquaculture, accounting for 34% and 35%, respectively, in 2019. Europe, Latin America, and the Middle East used 9%, 11%, and 7% of fishmeal in their aquaculture, respectively (EUMOFA, 2021).

The pig industry was the second-largest consumer of fishmeal, comprising 14% of total consumption in 2019, a decrease from 25% in 2009. Fishmeal's usage in poultry feed remained consistent, accounting for 5% of total consumption in 2019 (Figure 6). The intensifying competition for fishmeal between aquaculture and livestock producers has driven it to seek improved efficiency in its use. With the projected substantial and sustained growth of aquaculture in the coming years, the demand for fishmeal in this sector is expected to continue rising. Consequently, only feed-efficient and high-valued aquaculture products will likely be profitable with such inputs (EUMOFA, 2021).



Source: IFFO

Figure 6. The global distribution of fishmeal utilization across different sectors from 2009 to 2019.

European fishmeal and fish oil prices strongly correlate with global prices, largely influenced by the supply situation in South America (Peru and Chile) and the demand from Asia, particularly China. Over the period from January 2009 to January 2021, European fishmeal prices witnessed a 37% increase, reaching 1.164 EUR/tonne. During the same period, fish oil prices experienced a more substantial rise of 85%, reaching 1.419 EUR/tonne. The price surge for fishmeal and fish oil is attributable to declining supply and increasing demand, given the valuable nutritional contribution these ingredients offer to feed formulations. The expansion of the global aquaculture industry has favored species that require low inclusion rates of marine ingredients or command higher market prices, such as salmon and shrimp (EUMOFA, 2021).

In 2020, Peruvian fishmeal and fish oil production increased compared to the previous year, with over 1 million tonnes of fishmeal and around 165,000 tonnes of fish oil. This augmented supply from Peru was crucial in balancing the global fishmeal and fish oil market and helping stabilize prices. Throughout the latter half of 2020 and the first months of 2021, fishmeal prices in Europe displayed an upward trend and remained stable for the rest of the year. On the other hand, fish oil prices fluctuated between 1.993 EUR/tonne in June 2020 and 1.355 EUR/tonne in February 2021 (EUMOFA, 2021). As aquaculture production grows, fishmeal and fish oil production are expected to remain stable or slightly increase, focusing on utilizing byproducts and exploring alternative raw material sources like algae, krill, and insects through new investments.

Various alternatives, such as plants, insects, and terrestrial animal byproducts, have been thoroughly studied with a focus on sustainability and maintaining optimal nutrition for fish species like gilthead seabream and European seabass. However, despite the advancements, detailed knowledge of fish nutritional demands and the potential impacts of alternative ingredients on fish physiology and performance remain crucial for ensuring the success and sustainability of the aquaculture industry. Continuous research and development are essential to meeting the increasing demand for aquaculture products while minimizing the dependence on traditional fishmeal sources and promoting a more environmentally friendly and economically viable aquafeed industry.

Despite the positive results and promise shown by several alternative protein sources, the aquaculture industry continues to face a significant challenge in feed cost. Companies strive to develop more cost-effective formulations by reducing fishmeal and fish oil levels while maintaining growth, feed efficiency, survival, and other essential productive parameters (Hardy, 2010). Although partial fishmeal replacements have been relatively straightforward to achieve, complete or very high replacements have presented several problems, including reduced growth, altered metabolism, and negative impacts on health status (Bell & Waagbø, 2008; Kaushik *et al.*, 2004; Montero & Izquierdo, 2010). As a result, achieving complete or near-complete fishmeal replacement in aquafeeds without compromising the overall performance and health of the cultured species remains an ongoing challenge for the industry. Further research and innovation are necessary to overcome these obstacles and pave the way for more economically viable and sustainable aquafeed formulations.

2.1 Organic Aquaculture: Sustainable Nutrition

Organic aquaculture represents a distinct approach to production (Cottee & Petersan, 2009), driven by the growing emphasis on sustainable resource utilization (Mente *et al*., 2012, 2011). The ongoing discussion surrounding organic feeds for organic aquaculture stems from the need to balance the core principles of organic farming and the practical availability of feed sources for aquafeeds. The nutrition of organically raised aquatic animals entails feeding in a manner that allows for natural food intake, ensuring the animals' developmental, physiological, and behavioral needs are met (EC, 2007, 2009, 2014). Furthermore, feeds must align with the nutritional requirements of the cultured organisms, promoting growth and health, maintaining high product quality, and minimizing environmental impact (EC, 2007, 2009, 2014).

In inland water systems (extensive), such as ponds and lakes, cultured aquatic animals should primarily rely on naturally available food materials, including aquatic plants, algae, plankton, small invertebrates, and detritus (EC, 2009). For semi-intensive production, where increased nutrient availability is needed, natural food productivity can be enhanced through certified organic external inputs like fertilizers of both inorganic and organic origin (e.g., livestock manures, plant material, and inorganic nutrients), subject to organic regulations (EC, 2007, 2009). If it is necessary to introduce complementary feed or substances of natural origin, comprehensive documentation and evidence of the need must be provided (EC, 2007, 2009).

For omnivorous-carnivorous species like penaeid shrimps and freshwater prawns, the EU regulation stipulates that up to 25% of fishmeal and 10% of fish oil from sustainable fisheries can constitute their supplementary organic feed (EC, 2009, 2013, 2014). Catfish's feed ration may include a maximum of 10% fishmeal or fish oil from sustainable sources. Bivalve molluscs, filter-feeding organisms, are expected to obtain most of their natural nutrition, particularly when reared in hatcheries or nurseries. Their growth areas should exhibit high ecological or environmental quality (EC, 2009, 2014).

In intensive aquaculture setups, feed in pellet form is provided to match the nutritional needs at different developmental stages (Mente *et al*., 2011). Concerns about fishmeal and fish oil consumption in aquaculture feeds have grown due to industry expansion and declining wild stocks. Fishmeal and fish oil use contradicts organic sustainability principles, but salmon farming can now be a net producer of these components (Crampton *et al*., 2010). Genetically modified organisms (GMOs) and their derivatives are incompatible with organic production principles (EC, 834/2007). Synthetic feed ingredients, excluding essential additives, are prohibited, and synthetic antioxidants are not allowed.

In compliance with EU priorities, organic feed for carnivorous aquaculture animals adheres to specific regulations outlined in regulation (EU) 2018/848, governing organic production and the labeling of organic products. This legislation, emanating from the European Parliament and the Council of the European Union, meticulously defines standards applicable to the production, certification, and use of labels and advertisements related to organic production. Concerning feed for carnivorous aquaculture animals, as stipulated in paragraph 3.1.3.3 of the regulation, the following priorities must be considered:
a) Utilization of organic feed from aquaculture; b) Incorporation of fishmeal and fish oil derived from byproducts of organic aquaculture, sourced from fish, crustaceans, or mollusks; c) Inclusion of fishmeal, fish oil, and feeds from fish derived from byproducts of fish, crustaceans, or mollusks already caught for human consumption in sustainable fisheries; d) Integration of fishmeal, fish oil, and feeds from fish derived from whole fish, crustaceans, or mollusks caught in sustainable fisheries and not designated for human consumption; e) Incorporation of organic plant or animal origin feedstocks, with plant raw materials not exceeding 60% of the total ingredients.

Moreover, specific standards for feeds tailored to certain aquaculture animals are outlined in requirement 3.1.3.4 of the regulation. During the growth phase, freshwater fish, penaeids, freshwater shrimps, and tropical freshwater fish should be fed in the following manner:
a) Utilization of feeds naturally available in ponds and lakes; b) When natural feeds mentioned in point a) are insufficient, organic feeds of plant origin, preferably produced on the farm itself, or algae may be employed. Operators are required to maintain documentary evidence justifying the necessity of additional feeds; c) When natural feeds are supplemented according to point b): i) The feed ration for penaeids and freshwater shrimps (*Macrobrachium spp*.) may contain a maximum of 25% fishmeal and 10% fish oil derived from sustainable fishing; ii) The feed ration for fish of the genus Pangasius spp. may consist of a maximum of 10% fishmeal or fish oil derived from sustainable fishing. These regulations aim to ensure organic diets meet nutritional needs, enable natural feeding behaviour, minimize feed loss, and comprise natural, organic, and

sustainable components.

2.2 Advancing Sustainable Feeding in Organic Aquaculture: Advancements and Challenges in Substituting Fishmeal

There remains a need to discover new sources of proteins and lipids for organic aquaculture feeds, alongside reducing fishmeal and fish oil usage in these feeds. This requirement arises from the slowdown in wild fisheries. Nevertheless, the focus on the quality of alternative ingredients and their certification for use in organic aquaculture has intensified. Ongoing research is dedicated to examining new specially developed alternative components and evaluating the quality of the resultant products (Estévez et al., 2023; Di Marco et al., 2017; Carminato et al., 2020). These efforts aim to effectively tackle the complexities of producing organic feeds.

This characteristic promotes effective utilization by the fish while minimizing the release of nutrients into the environment. Consequently, substituting fishmeal for other protein source for fish species in organic farming diets is not simple. This complexity arises from fishmeal's distinctive attributes, including its elevated protein content, exceptional amino acid profile, high nutrient digestibility, strong palatability, substantial micronutrient content, and the general absence of anti-nutrients (Gatlin III *et al.*, 2007; Kaushik & Seiliez, 2010; Krogdahl *et al.*, 2010; Lund *et al.*, 2011, 2013; Macusi *et al.*, 2023). Furthermore, seabass, and seabream exhibit heightened protein requirements due to their notably carnivorous nature (Oliva-Teles, 2000; Peres and Oliva-Teles, 2009).

3. Exploring Diverse Feed Alternatives for Sustainable and Organic Aquaculture

Obtaining organic feed alternatives for organic aquaculture can be challenging due to several factors as mentioned in the previous sections. Firstly, sourcing organic ingredients that meet certification standards can be difficult, as they need to be grown or produced without the use of synthetic pesticides, fertilizers, or GMOs. Additionally, the availability of certified organic feed ingredients may vary depending on geographic location and seasonal factors.

Secondly, the cost of organic feed is often higher compared to conventional feed, making it less economically feasible for aquaculture producers, especially small-scale or low-income operations. The higher cost of organic feed can be attributed to the limited supply of organic ingredients, increased production costs, and additional certification requirements.

Furthermore, the formulation of organic feeds that meet the nutritional requirements of different aquatic species can be complex. Balancing the protein, lipid, carbohydrate, vitamin, and mineral content in organic feeds to support optimal growth and health of farmed organisms requires careful consideration and expertise. Overall, while progress has been made in developing organic feed alternatives for aquaculture, challenges remain in terms of availability, affordability, and formulation. Efforts to address these challenges through research, innovation, and collaboration are essential for the continued growth and sustainability of organic aquaculture.

The progression of aquaculture necessitates the integration of alternative ingredients to replace fishmeal (Gatlin III *et al*., 2007). These substitutes should meet specific criteria:

1. **Technical Viability:** Readily available, cost-competitive, and easy to handle throughout procurement, shipping, storage, and processing.
2. **Nutritional Attributes:** High protein content, a balanced amino acid (AA) profile resembling fish requirements, high nutrient digestibility, minimal anti-nutritional factors (ANF), low fiber and starch levels, and favorable palatability (Gatlin III *et al*., 2007).

In addition to plant-based proteins, there are several potential alternatives for aquaculture feed ingredients, including terrestrial animal byproducts (e.g., PAP and blood meal), and insect larvae/pupae. These candidates have the potential to replace fishmeal in aquaculture feed, as highlighted in studies by (Chary *et al*., 2023; Gasco *et al*., 2023; Sørensen *et al*., 2011; van Huis and Oonincx, 2017).

Processed animal protein (PAP) is a vital component in feeds, offering a valuable means of utilizing animal byproducts (Karapanagiotidis *et al*., 2019). The nutritional value of rendered animal protein ingredients is influenced by factors such as composition, freshness of raw materials, and processing conditions. PAP's high nutritional value makes it a favorable substitute for imported proteins like soy. Notably, its protein content (ranging from 45% to 90% on a fed basis) surpasses that of plant-derived feed ingredients. PAP contains around 10% phosphorus, a relatively lower proportion than the amino acid content. Blood meal also boasts high protein content (up to 80% in whole blood) and excellent protein digestibility (Bureau *et al*., 1999). It's rich in lysine and histidine but relatively low in isoleucine (Breck *et al*., 2003; El-Haroun & Bureau, 2007).

Despite potential concerns about the transmission of prions, the European Food Safety Authority (EFSA) issued a scientific panel opinion in 2011 asserting that processed animal protein in non-ruminant food-producing feed, adhering to the proposed intraspecies recycling ban, poses minimal human health risk (EFSA, 2011). In addition, Commission Regulation (EU) No. 56/2013 has established guidelines to prevent, control, and eradicate certain transmissible spongiform encephalopathies.

Another avenue being explored is the use of insects as a protein source in fish and crustacean diets (Alfiko *et al*., 2022; Cummins *et al*., 2017; Gasco *et al*., 2018; Iaconisi *et al*., 2017; Röthig *et al*., 2023; Rumbos & Athanassiou, 2019).

3.1. Exploring plant-based protein sources

Plant protein sources (PPS) emerge as promising candidates meeting technical requisites, often surpassing fishmeal in availability and cost. Successful partial replacement of fishmeal with various PPS has been demonstrated for carnivorous species (Table 2) (Nengas *et al*., 1996; Robaina *et al*., 1995). While processed animal proteins exhibited potential, their use was restricted in the European Union from 1999 to 2013 due to concerns about bovine spongiform encephalopathy (BSE) (Moutinho *et al*., 2017). Consequently, plant sources gained prominence, offering competitive prices and, after reauthorization, currently constitute 1% of global aquafeed ingredients (Coutinho *et al*., 2017). Continuous exploration and optimization of these alternatives are crucial for sustainable and economically viable aquafeed formulations.

Table 2. Some effects of plant protein meal dietary inclusion on some farmed fish

Ingredients	Fish species	Inclusion level	Effects	References
Plant proteins supplemented with lysine	Rainbow trout	50%	Improved growth performance, feed conversion ratio and survival.	(Cheng *et al*., 2003)
Corn gluten meal, wheat gluten, extruded wheat, soybean meal and rapeseed meal.	European sea bass	95%	No adverse effect on somatic growth or nitrogen utilisation.	(Kaushik *et al*., 2004)
Mixture of plant protein sources	Gilthead sea bream	75%	Growth performance was not affected.	(De Francesco *et al*., 2007)

Mix of soybean meal, soy protein concentrate and wheat gluten meal	Atlantic cod	50%	Growth was hardly affected.	(Hansen *et al*., 2007)
Combination of soybean meal and canola meal	Pacific white shrimp	80%	Not affected the growth performances.	(Suárez *et al*., 2009)
Mixture of plant proteins	Cobia	94%	No changes in the growth performances compared to fish meal diets.	(Salze *et al*., 2010)
Mixture of soybean meal, wheat gluten meal and corn gluten meal	Turbot	52%	Did not reduce the feed intake.	(Bonaldo *et al*., 2011)
A combination of pea, horse-bean and rapeseed	Rainbow trout	44%	No negative performances on growth.	(Lund *et al*., 2011)
Mixture of corn gluten meal, rapeseed meal, sorghum and wheat gluten	Black tiger shrimp	25%	No adverse effect on shrimp performances.	(Richard *et al*., 2011)
Cotton seed meal, sunflower meal and corn meal	Grass carp	75%	No adverse consequence in somatic growth and nitrogen utilization.	(Köprücü & Sertel, 2012)
Corn gluten meal	Hybrid sturgeon	55%	Did not affect the growth and FCR with 30 % of feed price reduction as compared to fish meal diets.	(Sicuro *et al*., 2012)
Mixture of soybean meal and canola meal	Kuruma shrimp	50%	No adverse effects on growth, feed utilization, body composition and nutrient utilization.	(Bulbul *et al*., 2013)
Mixture of plant protein sources with EAAs	Senegalese sole	75%	No impairments on feed intake, growth performance and protein utilisation.	(Cabral *et al*., 2013)
Mix of soy protein concentrate and barley protein concentrate	Red drum	50%	No effect on the growth performance, condition indices and whole-body composition.	(Rossi Jr *et al*., 2013)
Mixture of soybean meal, soybean protein concentrate and wheat gluten meal	Senegalese sole	30%	No changes in the growth performances as compared to fish meal diets.	(Rodiles *et al*., 2015)
Defatted rubber seed meal	Common carp	50%	No negative effect on the growth and feeding performances.	(Suprayudi *et al*., 2015)

Fish meal combined with mixture of plant proteins	Turbot	50 %	Positively affected the growth performance and welfare status.	(Bonaldo *et al.*, 2015)
Mix of fermented soybean meal, corn gluten meal and cottonseed meal with lysine	Chinese sucker	30%	No adverse effects on growth performance, body composition and digestive enzyme activities.	(Yu *et al.*, 2014)
Mix of soybean protein concentrate and corn protein concentrate	Shortfin corvina	75%	No compromising effect on growth performance.	(Minjarez-Osorio *et al.*, 2016)
Blend of soybean meal, peas, corn gluten, and wheat	Senegalese sole	75%	Growth performance was not impaired.	(Valente *et al.*, 2016)

Adapted from Daniel. (2018).

Drawbacks and Limitations of Plant Protein Sources

While plant protein sources (PPS) exhibit considerable potential, their utilization at high levels, especially in aquafeeds for carnivorous fish, comes with inherent limitations (Barrows *et al.*, 2008; Burr *et al.*, 2012; Gatlin III *et al.*, 2007; Hardy, 2010; Oliva-Teles *et al.*, 2015). These limitations pose challenges that researchers actively address:

1. **Nutritional Limitations:** Many PPS have relatively low protein content, restricting their use in high-energy diets. Additionally, their essential amino acid (AA) profile may significantly differ from the composition required by fish, with amino acids often being more limiting factors such as methionine and lysine (Hardy, 2010). The amino acid (AA) profile is of crucial importance, not only for fish growth but also for maintaining health status, supporting antioxidant defense mechanisms, and regulating overall metabolism (Kiron, 2012).
2. **Micronutrient Deficiencies:** Fishmeal contains essential vitamins, trace minerals, and biologically active compounds vital for fish nutrition. However, these are often lacking in vegetable meals, potentially leading to nutritional imbalances (Hardy, 2010).
3. **Anti-nutritional Factors (ANF):** PPS frequently contain ANFs, including non-starch polysaccharides (NSP), which can disrupt intestinal homeostasis. This disruption may result in histopathological alterations and inflammatory responses in fish (Krogdahl *et al*., 2003).
4. **Palatability Concerns:** The palatability of plant feedstuff could limit the incorporation of high levels of vegetable sources in aquafeeds, influencing fish intake negatively (Papatryphon & Soares Jr, 2001).

Understanding and mitigating these limitations are crucial for optimizing the inclusion of plant protein sources in aquafeed formulations and ensuring the overall success and sustainability of aquaculture practices.

Organic plant-based protein sources can serve as alternatives to fishmeal in aquafeeds, offering sustainable and environmentally friendly options. Some of the organic plant-based protein sources commonly used include organic soybean meal, organic wheat, and organic pea protein. Studies, such as the one conducted by Estevez and Vasilak (2023), have evaluated the efficacy of organic plant-based protein sources in aquafeeds. These studies assess factors such as nutrient composition, digestibility, growth performance, and feed conversion efficiency to determine the suitability of alternative protein sources for aquaculture. Producers can reduce their reliance on fishmeal by incorporating organic plant-based protein sources into aquafeeds while promoting sustainable and environmentally friendly aquaculture practices.

3.2. Exploring terrestrial animal byproducts (ABP) sources

For several years, processed animal byproducts (ABPs) have served as a valuable and feasible protein source in animal feed, including aquaculture feeds for various species. Aquaculture has a long history of utilizing ABPs, such as poultry meat meal, feather meal, meat and bone meal (MBM), whole dried blood, and plasma proteins in feeds for fish species like gilthead seabream, rainbow trout, Australian silver perch, African catfish, and tilapia, among others (Table 3) (Bureau *et al*., 1999, 2000; Fasakin *et al*., 2005; Nengas *et al*., 1999). A recent review by Glencross *et al*. (2020) highlighted the viability of processed animal proteins (PAPs) from land animals for aquaculture feeds. It emphasized their differences from plant protein sources like soybean meal. The rendering industry in Europe has made significant advancements in processing technology, improving the value, effectiveness, and safety of PAPs for aquaculture use.

Processed ABPs offer nutrient profiles that competitively contribute to meeting the essential requirements of farmed fish species. Animal proteins provide balanced protein, essential amino acid profiles, and bioavailable macro and trace elements like calcium, phosphorus, magnesium, iron, manganese, zinc, and copper (Moura *et al*., 2018). Poultry byproducts meal boasts an amino acid composition that resembles fishmeal, making it an attractive replacement for fish and soybean meal. Notably, poultry byproductsmeal and spray-dried poultry plasma were found to be substantial sources of carnosine and anserine, exhibiting antioxidative activity that can protect high-lipid aquafeeds susceptible to oxidation (Kohen *et al*., 1988; Wu *et al*., 2003) as reviewed by Li and Wu, (2020).

The commitment to organic principles extends to products of animal origin within the organic food and feed sector. By prioritizing the utilization of byproducts, organic aquaculture seeks to optimize resource efficiency, minimize waste, and enhance overall sustainability, embodying a conscientious approach to food and feed production.

Terrestrial meat and bone meal

Utilizing terrestrial animal byproducts (ABPs) as a protein source in aquafeeds involves processing minced ABPs through indirect heating in batch or continuous systems, following Method 1 described in Commission Regulation EC 142/2011, Annex IV, Chapter 3 (EU 142/2011). Although poultry byproducts have received the most attention in aquaculture, substantial research outside the EU uses terrestrial ABPs from species like cattle, sheep, and pigs. However, there have been mixed reviews on the effective digestibility of these products, with varying impacts on different fish species.

For instance, Allan *et al*. (2000) found that the apparent digestibility of MBM in Australian silver perch diets was only 55.4%, lower than fishmeal (76.8%–93.9%). Additionally, specific amino acids like lysine and methionine were reduced by up to 35.1% and 55.5%, respectively, compared to conventional fishmeal. MBM significantly reduced growth rates at more than 45% protein content in large yellow croakers, but no significant effects were observed at 45% or lower (Ai *et al*., 2006). For rainbow trout, dietary inclusion of up to 24% MBM was feasible without compromising growth, although a slight reduction in feed efficiency was noted (Bureau *et al*., 2000). It is essential to consider the balance of amino acid profiles and protein digestibility when formulating diets to avoid inaccuracies and imbalances between different feed ingredients.

While terrestrial ABPs offer nutrient profiles competitive with fishmeal, the quality, and composition can vary significantly between processing plants, impacting protein content and amino acid profiles (Hendriks *et al*., 2002). Consequently, MBM is deemed less suitable for aquafeeds due to this variation. Moreover, the BSE outbreak and the subsequent EU legislation banning MBM use in 2001 have limited research on terrestrial ABPs in aquafeeds, particularly within EU publications.

Although porcine PAPs are approved for use in EU aquafeeds (as non-ruminant PAP) by Commission Regulation (EC) No 51/2013, their use in European aquaculture, such as salmonids, is restricted due to the lingering social stigma from the BSE outbreak. Nonetheless, research has demonstrated the successful inclusion of porcine meals in several fish species, including replacing fishmeal in Nile tilapia diets (Hernández *et al.*, 2010). Pet food-grade porcine meal at a 34% dietary inclusion level achieved similar weight gain to sardine fishmeal in tilapia diets (Hernández *et al.*, 2010). Another study showed that channel catfish fingerlings could be fed with a diet containing up to 32% porcine meal without significantly affecting weight gain. However, the feed conversion ratio increased slightly (Li *et al.*, 2020).

While terrestrial ABPs show promise as aquafeed ingredients, their varied quality, social stigma, and potential FCR increases must be considered in determining their practical application in aquaculture. Animal byproducts (ABPs), such as feathers and poultry meal, are also explored as potential protein sources for aquafeeds. These terrestrial ABPs have gained attention in aquaculture due to their availability, cost-effectiveness, and potential to replace fishmeal in feed formulations.

Poultry meal

Poultry meal comes in different quality grades, including pet food grade, feed grade, and low ash. Each grade has unique characteristics, mainly related to the specific animal byproducts used and their nutritional qualities for different markets, such as pet foods or aquafeeds. The typically mixed species poultry ABP contains minced heads, feet, carcasses, and internal organs (excluding feathers), processed through continuous rendering methods. The resulting dried poultry meal generally contains 56%–62% protein and 11–17% lipid content.

Combining poultry meal with a small quantity of blood meal (2.5%) in aquafeeds has shown effectiveness in replacing 50% of the fishmeal component in the diet for Atlantic salmon without compromising growth performance (Hatlen *et al.*, 2015). This complementary use enhances the synergy of essential amino acids, providing superior biological value. In a study with totoaba, a marine fish species, replacing 67% of fishmeal with poultry meal resulted in a substantial increase in weight gain and survival compared to the control diet. However, when 100% of fishmeal was replaced with poultry meal, weight gain decreased significantly, indicating that complete substitution may deviate from the ideal protein profile found in fishmeal (Zapata *et al.*, 2016).

Research has shown that poultry meal yields higher growth performance indicators than other products like MBM (Saadiah *et al.*, 2011; O. Yildirim *et al.*, 2009; Q.-C. Zhou *et al.*, 2011), likely due to its more consistent amino acid profile and protein content. Lysine and methionine, critical factors for fish growth, are typically found at higher poultry meal concentrations than MBM. (Hendriks *et al.*, 2002). Despite this, poultry meal can also lead to amino acid imbalances that affect fish performance. For instance, African catfish-fed diets with certain poultry meal inclusion levels exhibited lower growth rates. Still, this negative impact was reversed when lysine was added to restore a more balanced essential amino acid profile (El-Husseiny *et al.*, 2018).

Similarly, black seabass experienced compromised growth performance when poultry meal replaced over 50% of fishmeal (Dawson *et al.*, 2018). On the other hand, cobia showed an enhanced protein efficiency ratio at an optimal poultry meal inclusion level of 30.75% (Zhou *et al.*, 2011). This indicates a defined ratio between major proteins in fishmeal and poultry meal that leads to an ideal protein balance diet. Synergistic responses were also observed in other fish species, such as gilthead seabream when using specific inclusion levels of poultry byproductsmeal instead of fishmeal.

Feather meal

Another ingredient that has been studied a lot is feather meal. Poultry feathers undergo a transformation into hydrolyzed meal since unprocessed poultry feathers are indigestible for single-stomach animals. Mixed poultry feather meal, derived from various poultry species, undergoes steam pressure processing to achieve a protein content of up to 85%. However, concerns arise regarding the digestibility of its protein, predominantly scleroprotein or keratin, constituting over 90% (Stone, 2009). Aquafeed trials incorporating feather meal up to 25%-30% inclusion levels have shown either equal or improved fish growth. Beyond this threshold, negative growth performance may occur due to amino acid deficiencies, particularly lysine and methionine, and the presence of poorly digestible fibrous keratin. Shorter gastrointestinal tracts in some fish species may exacerbate digestibility issues, prompting consideration of exogenous enzyme supplementation. Studies, exemplified by juvenile olive flounder demonstrate that crystalline amino acid supplementation enhances feather meal's nutritive value, supporting growth and feed utilization (Hasan *et al.*, 1997).

The use of feather meal in organic aquaculture is subject to regulations and standards set forth by organic certification bodies and governing agencies. Feather meal, derived from poultry feathers, can be a potential protein source for aquaculture feeds, including organic aquaculture, under certain conditions.

In organic aquaculture, the use of feed ingredients such as feather meal must comply with organic standards, which typically prohibit the use of synthetic chemicals, antibiotics, genetically modified organisms (GMOs), and other non-organic inputs. Feather meal itself is a byproduct of the poultry industry and can be considered an organic input if the poultry from which it is derived is raised according to organic standards. However, there are considerations to keep in mind regarding the use of feather meal in organic aquaculture such as the quality and Processing: Feather meal must be processed in a manner that does not involve the use of synthetic chemicals or other prohibited substances. Organic certification bodies may have specific requirements regarding the processing methods used to produce feather meal for organic use.

Table 3. Some effects of terrestrial byproducts meal dietary inclusion on some farmed fish

Ingredients	Fish species	Inclusion level %	Effects	References
Fermented feather	Tilapia	25–50	Decrease weight gain and associated growth parameters.	(Arunlertaree & Moolthongnoi, 2008)
Feather meal	Catfish	20	Feed intake dropped drastically 40-100%.	(Chor *et al*., 2013)
Poultry byproducts	Snapper	25	Growth performance was not affected.	(C. Hernández *et al*., 2015)
Poultry byproducts	Tilapia	50	Growth was hardly affected.	(Ayadi *et al*., 2012)
Poultry byproducts	European sea bass	60	Not affected the growth performances.	(Srour *et al*., 2016)
Poultry byproducts	Turbot	25	No changes in the growth performances compared to fish meal diets.	(Yigit *et al*., 2006)
Poultry byproducts	Tilapia	100	Did not affect the growth performances.	(Yones & Metwalli, 2015)
Blood meal	Catfish	50	No adverse effect on catfish growth performances and survival.	(Adewole & Olaleye, 2014)
Meat and bone meal	Snakehead	20	No adverse effect on growth and feed utilization.	(Yu *et al*., 2014)

3.3. Fishery and Aquaculture Byproducts

The remnants of captured and farmed aquatic organisms encompass components such as heads, fins, scales, skin, bones, and viscera. Similarly, shellfish and crustaceans contribute to byproducts like carapax, exoskeletons, shells, and debris, typically discarded during processing procedures like filleting, canning, and packaging for human consumption. Although laden with macro and micronutrients, these byproducts are often underutilized, leading to economic and environmental quandaries (W. Li *et al*., 2019; Olsen *et al*., 2014). The global fisheries have witnessed discards amounting to over 20 million metric tons per year, as estimated by the FAO, prompting a call to reassess their utilization (FAOSTAT, 2014). The European Union (EU) contributes around 5.2 million metric tons of byproducts yearly from its fishing sector (Lopes *et al*., 2015). The EU has enforced the landing obligation to curb unwanted catches, with complete implementation in January 2019. While this measure aims to reduce by-catch, specifically "non-target" fish, predicting the precise impact remains challenging due to disparate discard data and variability across the Mediterranean and Black Sea areas (Guillen *et al*., 2018). The processing of fish and aquaculture products has also led to substantial waste generation, especially during fish filleting and shell removal processes (Sierra Lopera *et al*., 2018). Table 4 showed some studies of aquaculture by products.

Table 4. Some effects of aquaculture by products meal dietary inclusion on some farmed fish

Ingredients	Fish species	Inclusion level %	Days of feeding	Effects	References
Fish silage	Red tilapia	50	84	No significant difference (P > 0.05) in weight gain or specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER)	(Madage *et al*., 2015)
Shrimp head meal	Catfish	20	84	No adverse effect on feed digestibility, mean weight gain, feed conversion ratio, protein efficiency ratio, and specific growth rate.	(Nwanna *et al*., 2004)
Krill meal	sturgeon	30	200	No negatively affecting growth, feed utilization, and body composition.	(Huang *et al*., 2016)

Strengths of Fish Byproducts

Nutritional Composition: The nutritional content of fish byproducts varies significantly based on the aquatic species and the specific tissue analyzed. For instance, yellowfin tuna skin contains approximately 32% protein (dry matter), 3% fat, and 63% ash. In Atlantic salmon heads, protein levels stand at 13%, lipids at 22%, and ash at 4%. Viscera of the same species show protein concentrations around 8%, lipid levels around 44%, and ash content at about 1%. In tilapia skeletons, protein content reaches 50%, lipids exceed 30% and ash is around 15% (Sierra Lopera *et al*., 2018). Protein and fat contents for anchovy byproducts (% DM) are 46% and 34% for the head, 41% and 25% for the frame, and 31% and 62% for viscera, respectively (Gencbay & Turhan, 2016) . Byproducts can account for 30% to 80% of unprocessed fish body weight and comprise muscle cuts (15–20%), skin and fins (1–3%), bones (9–15%), heads (9–12%), viscera (12–18%), and scales (5%) (Pinotti *et al*., 2016; Villamil *et al*., 2017). These discarded materials can be liquid (silage) or solid, posing hygienic challenges during storage and transportation.

Source of Protein and Nutrients: Incorporating discarded byproducts in aquaculture feeds has shown a potential to alleviate pressure on fish stocks used for fishmeal production, promoting sustainable aquaculture. Positive outcomes have been observed in fish and crustaceans when these byproducts substitute for fishmeal in diets containing high levels of plant proteins (García-Romero *et al*., 2014; Gisbert *et al*., 2018; Hernández *et al*., 2011; Uyan *et al*., 2006). Fish protein hydrolysates (FPH) derived from these byproducts have improved protein digestibility, attributed to the abundance of short peptides and free amino acids, enhancing palatability and absorption (Chalamaiah *et al*., 2012).

Bioactive Compounds and Feed Additives: Fishery and aquaculture discards, even after processing, can contribute to beneficial effects on fish immune systems. Shrimp shell discards have exhibited significant antioxidant activity when used to create hydrolysates, suggesting potential application in aquafeeds (Ambigaipalan & Shahidi, 2017). As feed additives, crab meal has enhanced flesh colour and sensory attributes in red porgy (*Pagrus pagrus*) (García-Romero *et al*., 2014). Shrimp shell meal inclusion (12-24%) for yellow croaker (*Larimichthys croceus*) has improved skin coloration and carotenoid levels without compromising growth and feed conversion (Yi *et al*., 2015). Novel Food and Feed Ingredients: Modern extraction techniques have recovered valuable nutrients, such as proteins, fatty acids, peptides, chitin, collagen, carotenoids, and minerals, from fish waste. These components hold significance in animal nutrition and human well-being (pharmaceuticals, cosmetics, nutraceuticals) (Bruno *et al*., 2019; Shabani *et al*., 2018). Long-chain n-3 polyunsaturated fatty acids extracted from oil-rich byproducts from various aquatic sources have applications in the food industry (Iriondo-DeHond *et al*., 2019).

3.4. Insect Meals as Alternative

Escalating fishmeal costs in aquaculture have triggered investigations into alternative protein sources, with insects emerging as promising candidates due to their rich nutritional composition. Insects offer abundant protein, amino acids, fats, minerals, and vitamins, exhibiting a comparatively lower environmental impact than conventional sources like fishmeal, whey, and egg proteins (Gasco *et al*., 2019; Lock *et al*., 2018; Smetana *et al*., 2019). The chemical composition of insects, influenced by rearing conditions, diet, and life stage, includes protein levels (50%-82%), amino acid profiles, lipid content (10%-30%), and variable fatty acid composition. Various insect species, including houseflies, black soldier flies, mealworms, and crickets, have been examined for their potential as feeds for different fish species. Partial replacement of fishmeal with insects generally yields positive outcomes, while complete substitution may lead to reduced growth and health (Henry *et al*., 2015). Recent research explores the use of insect meal, a protein-rich, flour-like ingredient, in dietary formulations. Investigations concerning freshwater and marine fish aim to understand the effects of different levels of fishmeal replacement with insect meal on feed digestibility and growth performance (Table 5). Variable results across studies underscore the need for further research, considering differences in species, life stages, insect meal inclusion levels, and feeding trial durations. The digestibility of insect-derived products depends on factors such as insect species, processing techniques, and levels of dietary incorporation. Chitin, a prevalent component of insects, can influence nutrient digestibility (Gasco *et al*., 2019).

Studies exploring digestibility across various fish species, such as mealworms (*Tenebrio molitor*, TM) and black soldier flies (*Hermetia illucens*, HI), have been extensively investigated, utilizing both full-fat and defatted meal options. Trials assessing the dietary inclusion of TM and HI species predominantly focused on growth performance, yielding heterogeneous outcomes. The inclusion of up to 16% TM meal resulted in enhanced specific growth rate (SGR) and weight gain (WG) in juvenile rockfish (Khosravi, Kim, *et al*., 2018). Similarly, juvenile mandarin fish exhibited improved growth rates up to 20% TM inclusion, followed by a decline at higher levels (Sankian *et al*., 2018). European seabass displayed comparable growth to FM-based diets at 25% TM inclusion, but growth declined at 50% TM inclusion (Gasco *et al*., 2016). Pearl gentian grouper experienced no growth impairment with 12.5% and 18.8% TM inclusion (Song *et al*., 2018). Trials with HI inclusion indicated that low to moderate levels of HI meal did not hinder fish feed conversion ratio (FCR) or growth performance. Rainbow trout, Atlantic salmon, Jian carp, and European seabass displayed unhindered growth with varying inclusion rates of HI meal (Belghit *et al*., 2019; Dumas *et al*., 2018; Magalhães *et al*., 2017; Renna *et al*., 2017; Stadtlander *et al*., 2017; J. S. Zhou *et al*., 2018). Despite these findings, the impact of insect meal inclusion on fish growth performance varies across species and inclusion levels, necessitating further research to establish optimal inclusion rates and conditions for different aquaculture applications.

Table 5. Some effects of insect meal dietary inclusion on some farmed fish

Fish species	Insect species	% Insect inclusion	Days of feeding	Effect on growth performance	Reference
European seabass	HI	6.5%, 13% and 19.5%	62	No effects on growth performance and feed efficiency. No effect on ADC of OM, DM, CP, AA.	(Magalhães *et al*., 2017)
	TM	25% and 50%	70	50% TM inclusion showed the lowest WG, FW, feeding rate and FCR. ADC of CP significantly higher than the control diet	(Gasco *et al.*, 2016)
Gilthead seabream	TM	25% and 50%	163	25% TM group had higher SGR, WG%, PER and lower FCR than other groups. ADC of CP and EE were lower in fish fed diets with 50% TM inclusion	(Piccolo *et al.*, 2017)
Rainbow trout	HI	6.6%, 13.2%, 26.4%	84	Good growth performance with all inclusion levels	(Dumas *et al.*, 2018)
	HI	20% and 40%	78	No effects on survival, FCR and growth performance	(Renna *et al.*, 2017)
	HI	28.1%	49	No effects on growth performance	(Stadtlander *et al.*, 2017)
	TM	8%, 16%, 24% and 32%	56	Increased growth until 16% TM inclusion. Then growth decreased at 24% and 32% TM inclusion	(Khosravi *et al.*, 2018)
Rockfish	HI	4.91%, 9.84% and 14.8%	114	Growth rate and FI increased and FCRunaffected by HI meal dietary Inclusion. No effect on ADC of CL, CP, AA and FA	(Belghit *et al.*, 2019)
Atlantic salmon	HI	60%	56	No effects on FCR and FI. Significant reduction in ADC of CL, CP and AA	(Belghit *et al.*, 2018)

TM: *Tenebrio molitor*; HI: *Hemetia illucens*; AA: Amino Acid; ADC: apparent digestibility coefficient; CL: crude lipid; CP: crude protein; DM: dry matter; OM: organic matter; EE: ether extract; FA: Fatty acids; GE: Gross energy; N: normal HI; E: fish offal-enriched HI; FM: fishmeal; FW: final weight; FI: feed intake; FCR: feed conversion ratio; PER: protein efficiency ratio; SGR: specific growth rate; WG: weight gain.

Justification and objectives

Justification

The aquaculture industry is facing increasing pressure to shift towards sustainable and organic practices to meet the growing demand for seafood while minimizing its environmental impact. One of the critical challenges in achieving this transition is the development of high-quality organic feeds that can replace traditional protein sources.

Until now, the research on plant or animal eco-ingredients for fish nutrition is very scarce, in part because the missing of organic animal products. For obtaining an aquaculture organic diet it is necessary to assay organic animal by-products, in order to reduce or eliminate the fish meal, which is not a organic ingredient.

General Objective

The overarching goal of this thesis is to develop 100% organic feeds for organic aquaculture, primarily focusing on seabass, rainbow trout, and seabream, among the most produced species in Spain and Europe. This research aims to contribute to the aquaculture sector's sustainable and environmentally friendly practices by replacing traditional fish meal with organic alternatives. Our goal is to obtain 100% organic diets, since fish meal is not strictly organic, and that is why animal sources are studied. The study will initially consider ingredients compliant with existing regulations and subsequently explore the inclusion of PATs of organic origin, such as poultry meal, Iberian pork, and insect meal.

By pursuing these objectives, this thesis provides valuable insights into the viability and efficacy of organic ingredients in aquaculture feeds, ultimately supporting the transition towards more sustainable and organic practices in the aquaculture industry. Additionally, the economic analysis will help assess the economic feasibility of adopting these organic feeds, thus contributing to the economic sustainability of organic aquaculture.

In this thesis study, two main objectives have been outlined:

1. The mapping of ingredients suitable for organic aquaculture.
2. Exploring the inclusion of organically sourced Terrestrial Animal Proteins (TAPs).

To derive specific objectives from these main objectives, consider the following:

From Main Objective 1: The mapping of ingredients suitable for organic aquaculture:

1. Identify and evaluate organic ingredients commonly used in aquafeed formulations.

2. Assess the nutritional composition and availability of identified organic ingredients.

3. Investigate the sustainability and environmental impact of selected organic ingredients.

4. Compare the performance of different organic ingredients in terms of growth, health, and feed utilization.

5. Develop a comprehensive database or guideline for selecting organic ingredients based on their suitability for aquaculture.

From Main Objective 2: Exploring the inclusion of organically sourced TAPs:

1. Determine the potential TAP sources suitable for organic aquafeeds (e.g., insect meal, poultry meal).

2. Evaluate the nutritional profile and quality of organically sourced TAPs compared to conventional alternatives.

3. Investigate the feasibility and efficacy of incorporating TAPs into organic aquafeed formulations.

4. Assess the impact of TAP inclusion on fish growth, health, and overall performance.

5. Analyze the economic viability and environmental sustainability of utilizing organically sourced TAPs in aquafeed production.

Chapter 1.

Effects of Eco-Organic Feed on Growth Performance, Biometric Indices, and Nutrient Retention of Gilthead Seabream (*Sparus aurata*)

Effects of Eco-Organic Feed on Growth Performance, Biometric Indices, and Nutrient Retention of Gilthead Seabream (*Sparus aurata*)

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Sustainability, 2023

Tefal, E.; Tomás-Vidal, A.; Martínez-Llorens, S.; Jauralde, I.; Sánchez-Peñaranda, D.; Jover-Cerdá, M. Effects of Eco-Organic Feed on Growth Performance, Biometric Indices, and Nutrient Retention of Gilthead Seabream (Sparus aurata). *Sustainability* 2023, 15, 10750. https://doi.org/10.3390/su151410750

Abstract

This study examined how eco-organic feed affects the growth performance, nutrient efficiency, feed utilisation, and body composition of gilthead seabream. Six different diets were tested, including a control diet (CONT) without organic ingredients and four diets with 100% organic ingredients: trout (TRO), seabass (SBS), poultry (POU), and mix (MIX), along with a control organic diet (ORG) containing organic ingredients and 30% fishmeal. The experiment lasted 70 days, and the fish were fed twice a day, starting with an initial weight of 60.5 g. The results showed that the highest growth rates were observed in fish fed the ORG and CONT diets containing fishmeal. Conversely, the POU diet resulted in the lowest growth rate, survival rate, and highest value for feed conversion ratio (FCR). Almost all essential amino acid efficiency values were high in fish fed the ORG and CONT diets. Still, significant differences were noted in the retention efficiency of fatty acids across all diets. The retention efficiency was higher in the CONT diet, followed by the ORG diet. However, the economic conversion rate was lower for CONT, SBS, TRO, and MIX. Overall, using organic diets of animal origin impacted the growth performance of gilthead seabream, but it is still a promising approach.

Keywords: *sustainable aquaculture; organic diets; amino acids; organic fish; organic production; fishmeal substitution*

1. Introduction

The improvement of aquaculture sustainability is an issue of increasing importance. The lack of fish meal has forced the aquaculture sector to seek a more reliable solution for the environment and fishery resources. Advances in aquaculture sustainability are gradually reducing the amount of wild fishmeal used in aquafeeds. These advances have manifested in various ways, such as creating standards and regulations for protecting the environment or creating or caring for the environment in production processes and reducing and managing waste [1]. Organic aquaculture is a term usually understood as synonymous with ecological aquaculture and is a comprehensive method of farming fish and other marine species that adheres to organic principles [2]. The exact definition of organic may vary depending on the certification system with specific rules regarding production methods, and only products that follow the guidelines are allowed to use certified organic labels.

In most organic systems, such as EU regulatory processes, the preference for organic consideration of the raw ingredients is for the use of byproducts coming from certified organic farms [3]. Organic aquaculture is a relatively new food-producing sector [4]. The first common carp (Cyprinus carpio) standard was established in Austria in 1994 [5]. According to the European Market Observatory for Fisheries and Aquaculture (EUMOFA) [6], overall organic aquaculture output in the EU 27 was estimated at 74,032 tonnes in 2020, accounting for 6.4% of the total EU aquaculture production. Production has grown by 60% from 2015 (46,341 tonnes at the EU 27 level in 2015), while European nations represent approximately 20% of the world’s organic aquaculture. However, some European nations have decreased production lately [7].

The fundamental organically produced species, arranged by significance, are salmon, mussels, carp, trout, seabass, and seabream [8]. Specifically, for organic aquaculture, the Regulation mentions that it is a relatively new production sector, and the number of aquaculture production units converting to organic production is expected to rise. This will generate new experience, technical knowledge, and advances in ecological aquaculture that must be reflected in production standards [9]. The scope of organic farming is still minimal regarding the primary Mediterranean-farmed species: seabass and seabream [10]. Organic feeds result from a farming system that does not use synthetic fertilizers, pesticides, growth regulators, or livestock feed additives. Organic legislation generally prohibits irradiation and using genetically modified organisms (GMOs) or products derived from or containing GMOs [11].

Organic seabream production has been hampered mainly by economic concerns, such as the higher cost of production feed prices, which have deterred consumers and producers [10]. According to recent consumer preference studies, the Mediterranean has much potential for organic seabream production [12,13]. Furthermore, expanding organic aquaculture production is limited by the need for more organic feed, particularly for carnivorous species. Indeed, the EU organic legislation imposes minimums on the source of organic ingredients for the formulation of nutritionally balanced diets [14,15]. Only some ingredients must be organically certified (60% in the EU); only the plant ingredients must be organic. The limitations of the protein ingredients that can be used for organic feeds are one of the main challenges. Currently, most organic labels are allowed to use non-organic fishmeal, although the use of fishmeal from sustainable fisheries is required. However, according to the regulation (EU) 2018/848 [16], transformed animal proteins (TAPs) can be used, and TAPs of organic origin would be considered organic. To reduce reliance on conventional fishmeal and fish oil, fisheries and aquaculture byproducts are an excellent sustainable aquafeed option [17,18].

Using byproducts from organic production would open the door to an increase in the percentage of the minimum organic raw ingredients used in the organic formulation. There are few studies in which organic feed has been used in carnivorous fish species. The regulatory limitations are summarised as all vegetable ingredients must be organic, a maximum of 60% of vegetable ingredients are allowed, and the absence of synthetic amino acids. Fish meal can be used as long as it comes from sustainability-certified fisheries or organic production. The use of non-synthetic amino acids is allowed. Therefore, the fundamental protein source with the scope of sustainability should be fishmeal from organic aquaculture or sustainable fisheries, and, due to the lack of availability, it is different. With these limitations, the availability of good ecological protein sources suitable for carnivorous fish is complicated. The main challenges for the supply of feed ingredients for the organic production of carnivorous fish are to increase the diversity of ingredients available to balance the amino acid profile without synthetic amino acids and to identify new sources suitable for the supply of eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3) [14].

Even fewer studies compare conventional feeding with organic feeding. Sardinha *et al.* [19] in seabream, Pascoli *et al.* [20] in seabass, or Di Marco *et al.* [21] in seabass (Dicentrarchus labrax) and seabream (Sparus aurata) compare organic with conventional feeding. However, in these studies, organic feeds have conventional fishmeal as a protein source. The stress and immunological markers in the fish were similar [20], while the fillet fatty acid composition varied depending on the diet [22]. The byproductsmeals used in this study need to be better studied. More protein sources from organic sources must be prioritised in research. The main alternatives are using TAPs from organic cattle and generating a circular economy, using the remains of other species of organic fish, and avoiding cannibalism to generate organic meals with nutritionally optimal fatty acids and amino acid profiles.

The purpose of this research was to specifically determine the effect that organic feeds have on the growth of gilthead seabream and to see how new organic raw materials, such as poultry, remains of trout, and remains of seabass, affect growth and nutritional and biometric parameters. The goal was to better understand organic feeds as alternative ecological sources for gilthead seabream. These products may have a role in developing organic aquaculture in the Mediterranean to make it more sustainable.

2. Materials and methods

2.1 Production System

The growth trial was carried out in a recirculating saltwater system (65 m^3 capacity), with a rotary mechanical filter and a gravity biofilter (about 6 m^3), and eighteen cylindrical fiberglass tanks (1750 L, three per treatment). Aeration was installed in all tanks at the Laboratory of Aquaculture (Animal Science Department at Universitat Politècnica de València, Valencia, Spain). The temperature was held constant at 21 ± 1 °C, the dissolved oxygen level was 8.7 ± 1.6 mgL^{-1}, the salinity was 33.3 ± 2.4 g L^{-1}, the pH was 7.49, nitrates were 104.2 mg L^{-1}, nitrites was 0.38 mg L^{-1}, and the ammonium level was 0.22 mg L^{-1}. The photoperiod was regular throughout the experiment, and all tanks had similar lighting conditions.

2.2 Fish and Trial Design

Organic seabream juveniles from the Sonrionansa S.L. fish farm located in Pesues (Cantabria) were used for the study. The fish were transported to Universitat Politècnica de València and distributed in experimental tanks. All fish were acclimated to laboratory conditions two weeks before the feeding experiment. At the start of the trial, all fish were weighed individually to calculate the initial body weight and the initial biomass in each tank. A group of 720 fish (average weight 60.5 g) was distributed in 18 experimental tanks with a stocking rate of 22.9 fish/m^3 (40 fish per tank). The test lasted 70 days; six diets were tested in triplicate, shown in Table 1: the control diet (CONT, without organic ingredients and 30% commercial fishmeal content), the organic control diet (ORG, with organic ingredients and 30% commercial fish meal content), and four diets with 100% organic ingredients: the TRO diet (with organic trout meal as a protein source), the SBS diet (with remains of organic seabass as a protein source), the POU diet (with organic poultry meal as a protein source), and the MIX diet (containing three equal parts of the feed from the organic remains of seabass, trout, and poultry). From Monday to Saturday, the fish were hand-fed twice daily (9:00 and 17:00 h) until apparent satiation. The fish were starved on Sunday. The pellets were progressively dispersed, enabling all fish to consume and the overall amount of feed distributed was recorded. Every 30 days, all fish were weighed. The fish were anesthetised with 10 mg L^{-1} clove oil (Guinama®, Valencia, Spain) containing 87% eugenol before being weighed, and were not fed the previous day.

Table 1. Ingredients and formulation of the experimental diets.

	CONT	ORG	TRO	SBS	MIX	POU	PRICE
Ingredients (g/kg DM)							EUR tonn^{-1}
Fish meal [1]	300	300					1933
Organic trout meal [2]			400		140		750
Organic seabass meal [3]				405	140		750
Organic poultry meal [4]					140	435	750
Wheat [5]	180						473
Organic wheat [6]		22	216	84	120	119	515
Organic pea [7]			111	54	118	102	550
Organic corn [8]		8					515
Organic spelled bran [9]		10					1950
Wheat gluten [10]	120						1750
Soybean [11]	214						746
Organic soybean [12]		504	150	433	270	272	1355
Fish oil [13]	50	67	50		50	50	3671
Soybean oil [14]	100						1558
Organic soybean oil [15]		59	53				1815
Calcium phosphate [16]	23	20					3500
Vegetable methionine [17]	3		10	14	12	12	2220
Vitamin and mineral mix [18]	10	10	10	10	10	10	5000
Price [19] (EUR kg $^{-1}$)	1.51	1.76	1.03	1.04	1.07	1.07	

[1] Fish meal (91.70% DM, 74.30% CP, 12.40% CL, 14.30% ash). [2] Organic trout meal (95.71% DM, 76.71% CP, 17.36% CL, 9.43% ash) (Naturix, Valderrebollo, Guadalajara). [3] Organic seabass meal (98.83% DM, 44.91% CP, 40.93% CL, 17.83% ash) (Andrómeda, España). [4] Organic poultry meal (97.48% DM, 58.45% CP, 27.50% CL, 16.06% ash). [5] Wheat (87.82% DM, 11.41% CP. 1.76% CL, 1.63% ash). [6] Organic wheat (92.40% DM 12.70% CP, 2.30% CL, 1.70% ash) (Piensos Montoya, Valencia). [7] Organic pea (91.80% DM 23.00% CP, 3.30% CL, 2.10% ash) (Piensos Montoya, Valencia). [8] Organic corn (86.40% DM 8.3% CP, 3.30% CL, 1.10% ash) (Piensos Montoya, Valencia). [9] Organic spelt bran (80.48% DM 17.98% CP, 0.61% CL, 4.26% ash) (Piensos Montoya, Valencia). [10] Gluten (93.33% DM 81.00% CP, 0.86% CL, 0.86% ash). [11] Soybean (88.13% DM 49.90% CP, 2.20% CL, 7.08% ash). [12] Organic soybean (92.30% DM 53.40% CP, 4.30% CL, 5.90% ash) (Piensos Montoya, Valencia). [13] Fish oil (Industrias Afines, S.L. (Arpo), Polígono industrial A Veigadaña, Rúa as Baloutas, de Abaixo, 24, 36416, Pontevedra). [14] Soybean oil (refined soybean oil, Casimiro Perez Sl, Gabriel Miró, 16, 18, 03804 Alcoi, Alicante). [15] Organic soybean oil (Clearspring Ltd., Acton Park Estate, London W3 7QE, United Kingdom). [16] Calcium phosphate. [17] Vegetable methionine (Adibio S.L., Edificio Galileo, C/ Enebros 74, 2ª planta, 44002 Teruel, España). [18] Vitamin and mineral mix (g kg^{-1}): premix: 25; choline 10; DL-a-tocopherol, 5; ascorbic acid, 5; $(PO4)_2Ca_3$, 5. Premix composition: retinol acetate, 1,000,000 IU kg^{-1}; calcipherol, 500 IU kg^{-1}; DL-a-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. [19] Prices in EUR kg^{-1}.

2.3 Diets and Feeding

Six diets, isoproteic (45%) and isolipidic (18.8%), were formulated (Table 1). The CONT and ORG diets included 30% non-organic fishmeal because no organically certified commercial fishmeal exists. The ORG diet differed from the CONT diet in using organic ingredients: in ORG, unlike in CONT, the use of non-organic ingredients was avoided to the maximum.

Four alternative diets (TRO, SBS, POU, and MIX) were also designed to avoid using non-organic ingredients. Vegetable methionine of sustainable origin was added to the organic diets to meet the needs of gilthead seabream because it is a limiting amino acid for growth [23]. The control di*et al*so had added methionine; however, this was of synthetic origin. The diets were prepared using a semi-industrial twin-screw extruder (CLEXTRAL BC 45, St. Etienne, France). A screw speed of 100 rpm, temperature of 110 °C, and pressure of 40–50 atm were the processing conditions. Conventional fish oil from sustainable fisheries was used because organic fish oil has not yet been certified.

2.4 Proximate Composition and Amino Acid Analysis

The raw materials were chemically analysed. At the start of the experiment, five fish were sampled, triturated, and homogenised before being analysed for chemical composition. At the end of the experiment, five fish per tank were randomly sampled to determine the biometric parameters and were triturated and homogenised before being analysed for the proximate body composition. The dietary ingredients and the whole body of fish fed the six experimental diets were analysed according to AOAC (1990) [24] procedures: dry matter (105 °C to constant weight), ash (550 °C to constant weight), crude protein (N × 6.25) using the Kjeldahl method after acid digestion (2300 Kjeltec Analyzer Unit), and crude lipid extracted with diethyl ether (ANKOM XT10) using the Dumas principle.

The diet (Table 2) and body fish amino acid content were analysed using a Waters HPLC system that included two pumps (Model 515; Waters), an autosampler (Model 717; Waters), a fluorescence detector (Model 474; Waters), and a temperature control module, as described by Bosch *et al*. [25]. After hydrolysis, an internal standard of aminobutyric acid was introduced. AQC (6aminoquinolylNhydroxysuccinimidyl carbamate) was used to derive the amino acids. After oxidation with performic acid, methionine and cysteine were identified as methionine, sulphone, and cysteine acid, respectively. The Waters AcQ isolated the amino acids using a C18 reverse-phase column with a 150 mm × 3.9 mm tag. All analyses were carried out in duplicate.

Table 2. Amino acid composition of experimental diets (g kg^{-1} DM).

	CONT	ORG	TRO	SBS	MIX	POU
EAAs						
Arginine	37.4	45.3	31.8	33.5	37.3	36.6
Histidine	13.9	14.7	9.7	9.2	11.2	11.0
Isoleucine	25.5	27.2	19.9	17.8	21.2	21.7
Leucine	43.0	44.5	32.9	30.0	34.9	36.9
Lysine	34.8	42.3	32.6	27.8	33.9	32.2
Methionine	12.9	9.8	9.9	8.0	9.9	9.8
Phenylalanine	27.7	27.8	20.0	18.9	21.3	21.9
Threonine	22.8	24.7	19.9	16.9	20.4	20.2
Valine	30.2	31.9	24.4	21.5	25.3	25.8
NEAAs						
Alanine	28.1	31.1	26.2	23.5	26.9	27.4
Aspartic acid	47.6	62.3	46.0	41.1	49.9	46.0
Cysteine	5.9	4.8	4.2	3.4	4.2	4.7
Glutamic acid	123.1	97.0	76.8	71.4	81.6	90.0
Glycine	29.3	31.1	35.2	30.0	33.8	32.7
Proline	37.3	28.1	25.1	23.5	26.4	28.2
Serine	24.9	26.8	21.8	20.4	22.9	22.0
Tyrosine	18.1	19.7	13.9	12.6	14.6	14.6
EAA/EAA	7.9	8.9	8.1	8.1	8.3	8.1

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

2.5 Growth and Nutrient Efficiency Indices

After the experiment, the growth and nutrient efficiency indices were calculated using the tank as the experimental unit. The specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), economic conversion ratio (ECR), survival (S), productive protein value (PPV), and productive fat value (PFV) were all determined, with consideration given to the monthly biomass reports of any deceased fish [26].

2.6 Biometric Indices

After the feeding trial, every fish was weighed on its own. Biometric indices were determined by randomly slaughtering five fish from each tank, fifteen per treatment, using a lethal bath of clove oil (150 mg L^{-1}). The samples collected from each tank were combined and kept at −30 °C for further analysis. The fish’s overall weight and length, as well as the weights of its internal organs, visceral fat, and liver, were measured to determine the condition factor (CF), viscerosomatic (VSI), visceral fat (VFI), and hepatosomatic (HSI) indices [26].

2.7 Fatty Acid Analysis

Total lipid fatty acid methyl esters (FAMEs) were produced directly, as stated by O'Fallon [27]. A focus gas chromatograph (Thermo, Milan, Italy) with a split/splitless injector and a flame ionisation detector was used to analyse the FAMEs. A fused silica capillary column SPTM 2560 (Supelco, PA, USA) was used to separate the methyl esters (100 m × 0.25 mm × 0.2 µm film thickness). Helium was used as the carrier gas, with a linear velocity of 20 cm/seg. A split ratio of 1/100 was used to inject the samples. The starting oven temperature was set at 140 °C for five minutes, and then increased to 240 °C at a rate of 4 °C/min for another 30 min. The temperature of the detector and injector were both set at 260 °C. Individual fatty acids were identified by comparing retention periods to Supelco-supplied fatty acid methyl ester standards. Only fatty acids with a minimum concentration of 0.1 percent were considered. To quantify the fatty acids, we calculated the g of fatty acids per 100 g of a sample using the sample weight data from the analysis and measured using C13:0 as an internal standard. Table 3 shows the fatty acid content of the trial diets.

Table 3. Fatty acid composition of the experimental diets (g kg^{-1} in DM).

	CONT	ORG	TRO	SBS	MIX	POU
SFA						
(C13:0)	3.7	3.8	3.5	3	3.5	4.3
(C14:0)	3.7	13.5	3.3	5.3	5.3	8.9
(C15:0)	0.4	0.7	0.4	0.6	0.5	0.6
(C16:0)	26.6	28.9	31.3	27.8	32	33.8
(C17:0)	0.5	0.5	0.5	0.5	0.6	0.6
(C18:0)	0.6	0.4	0.6	0.5	0.5	0.5
MUFA						
(C16:1)	4.2	5.3	4.2	6	6.2	6.2
(C17:1)	0.2	0.3	0.3	0.4	0.4	0.3
(C18:1n9c)	40.4	28.6	49.5	41	49.1	42.8
(C18:1(n-7)	3.8	2.3	4.3	4.3	4.7	3.8
(C20:1)	2.3	1.6	0.1	7	7.3	3.9
(C24:1)	0.6	0.4	0.6	0.7	0.7	0.7
PUFA						
(C18:2n6c) LA	63.1	26.2	57.6	40.5	42.9	42.6
(C18:3n3) LNA	9.3	5.2	7.9	6.9	6.9	6
(C20:2)	0.4	0.2	0.6	0.9	1	0.5
(C20:3n6)	0.1	0.1	0.2	0.2	0.2	0.2
(C20:4n6) ARA	0.8	0.4	1.9	0.7	1.3	1.2
(22:4n-6)	0.1	0	0	0	0	0
(22:5n-3)	0.1	0	0.2	0.1	0.1	0.1
(20:5n-3) EPA	6	3.5	4.5	5.7	5.1	4.6
(22:6n-3) DHA	9.9	6.1	6.8	11.8	10	7.7

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; LA:

linoleic acid; LNA: linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid.

2.8 Statistical Analysis

Growth data, feed utilisation, and all other data obtained were evaluated using analysis of variance (ANOVA), with the initial live weight as a covariate [28]. The Newman–Keuls test assessed specific diet differences at $p < 0.05$ (Statgraphics, Statistical Graphics System, Version Plus 5.1, Herndon, VA, USA). The tank means were the statis-tical unit.

2.9 Ethical Statement

This study followed European Union Council Directive 2010/63/ EU, which establishes the basic requirements for animal protection during experimentation, and Spanish state law (Spanish Royal Decree 53/2013), which governs animal use in experimentation and other scientific objectives. The Ethics Committee of the Polytechnic University of Valencia (UPV) approved the experimental methodology. The fish were examined every day. In addition, after sedation with clove oil dissolved in water (0.01 mg/L of water) to minimise animal suffering, the health state of the fish was determined through observation. An excess of clove oil (150 mg L^{-1}) was used to euthanise the animals, which were dissected.

3. Results

3.1 Fish Growth

Figure 1 shows the average growth of the gilthead seabream fed the different ex-perimental diets. The gilthead seabream fed the CONT and ORG diets obtained the highest average weight after the 70-day trial period ($p < 0.05$).

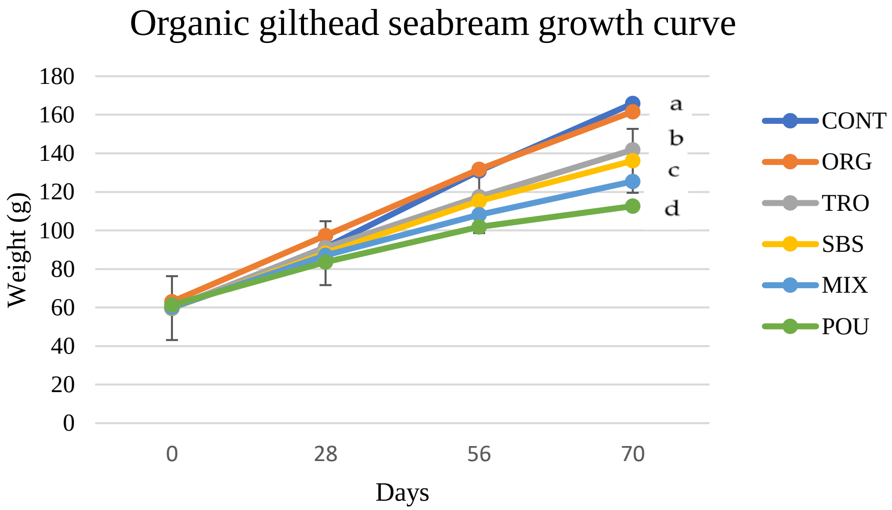

Figure 1. Evolution of the average body weight of gilthead seabream fed on the experimental diets

Values represented as mean ± standard error (n=3). Different superscript in each sampling means significant differences (*p < 0.05*). Test Newman-Keuls.

Regarding organic diets without fishmeal, the fish fed the TRO and SBS diets showed the highest growth ($p < 0.05$), followed by the MIX diet. Finally, POU exhibited the lowest growth. As reported in Table 4 and Figure 1, the best growth occurred in the two control diets containing 30% fishmeal, CONT and ORG (165.8 and 161.6 g, respectively), regardless of whether the rest of the ingredients included were organic. The fish that grew the best were those fed organic fish, TRO and SBS (141.8 and 136.1, respectively). The worst growth occurred with diets containing poultry meals, either alone (POU) or in a mixture (MIX) (125.4 and 112.6, respectively). Significant differences ($p < 0.05$) were observed in the SGR, showing the highest value in the fish fed the CONT diet (1.46%). Fish fed the POU diet presented the lowest value (0.87%).

Table 4. Overall performance of gilthead seabream fed the organic experimental diets.

	CONT	ORG	TRO	SBS	MIX	POU	SEM
Initial weight (g) [1]	59.5	63.0	59.9	59.7	60.1	61.3	1.30
Final weight (g)	165.8 ^{a}	161.6 ^{a}	141.8 ^{b}	136 ^{b}	125.4 ^{c}	112.6 ^{d}	3.80
Mortality (%)	3.3 ^{b}	4.2 ^{b}	5 ^{b}	7.5 ^{b}	8.2 ^{b}	13.3 ^{a}	1.44
SGR (% /day) [2]	1.46 ^{a}	1.35 ^{b}	1.23 ^{c}	1.18 ^{c}	1.05 ^{d}	0.87 ^{e}	0.02
FI(g/100gfish/day) [3]	2.25 ^{b}	2.32 ^{b}	2.65 ^{a}	2.50 ^{ab}	2.46 ^{ab}	2.57 ^{ab}	0.07
FCR [4]	1.7 ^{d}	1.9 ^{c}	2.3 ^{b}	2.3 ^{b}	2.4 ^{b}	2.9 ^{a}	0.08

ECR [5]	2.60 ^{b}	3.36 ^{a}	2.40 ^{b}	2.35 ^{b}	2.57 ^{b}	3.13 ^{a}	0.08

For each treatment, the values are represented as mean ± standard error (n = 3). The values that do not share the same letter differ significantly (p < 0.05). Newman–Keuls test. 1 Initial weight was considered covariable for final weight and specific growth rate. 2 Specific growth rate (SGR) = 100 × ln (final weight/initial weight)/days. 3 Feed intake (FI) (g 100 g fish−1 day−1) = 100 × feed consump-tion (g)/average biomass (g) × days. 4 Feed conversion ratio (FCR) = feed consumption (g)/weight gain (g). 5 Economic conversion ratio (ECR) = feed consumption (g) × price (EUR/kg)/weight gain (g).

Fish fed the POU diet exhibited the highest mortality (13.33%) ($p < 0.05$), while those fed the CONT, ORG, TRO, SBS, and MIX diets presented mortality rates (3.33 to 8.25%) without significant differences ($p > 0.05$). Regarding the feed conversion rate, fish fed the POU diet presented the highest value of FCR (2.92%), and fish fed the CONT and ORG diets showed the lowest (1.72% and 1.91, respectively) ($p < 0.05$). The fish fed diets containing organic fish remains (TRO, SBS, MIX) showed FCR without significant differences between them ($p > 0.05$). The TRO diet was higher (FI 2.65%) than the CONT and ORG diets (2.25 and 2.32, respectively), and the highest was in the TRO diet (2.65%) ($p < 0.05$). If the economic conversion rate is observed (ECR), the ORG and POU diets showed significant differences (3.36 and 3.13 EUR/kg, respectively) ($p < 0.05$) compared to the rest of the diets, which ranged from 2.35 to 2.60 EUR/kg.

3.2 Biometric Indices of Gilthead Seabream Fed Experimental Diets

The biometric indices after the trial period are presented in Table 5. No significant differences ($p > 0.05$) were found in any biometric parameters calculated according to the diet, except for the condition factor. The CF presented significant differences; fish fed the MIX diet had the lowest value (1.77%), and fish fed the ORG diet had the highest value (2.02%) ($p < 0.05$).

Table 5. Biometric indices of gilthead seabream fed the experimental diets.

	CONT	ORG	TRO	SBS	MIX	POU	SEM
VSI (%) [1]	6.1	9.0	5.5	6.6	5.9	6.4	0.87
HSI (%) [2]	1.2	0.9	0.8	0.8	0.8	0.9	0.16
VFI (%) [3]	1.6	1.9	1.2	0.9	1.0	0.9	0.47
CF (g/cm^3) [4]	2.0 ^{ab}	2.0 ^{a}	1.9 ^{abc}	1.9 ^{bc}	1.7 ^{c}	1.8 ^{bc}	0.13

For each treatment, values are represented as mean ± standard error (n = 9). The values that do not share the same letter differ significantly ($p < 0.05$). [1] Viscerosomatic index (VSI) (%) = (visceral weight (g)/total fish weight (g)) × 100. [2] Hepatosomatic index (HIS) (%) = (liver weight (g)/total fish weight (g)) × 100. [3] Visceral fat index (VFI) (%) = (visceral fat (g)/total fish weight (g)) × 100. [4] Condition factor (CF) (g/cm^3) = (total fish weight (g)/$length^3$ (cm)) × 100.

3.3 Proximate Body Composition and Nutrient Efficiency Retention

The results of the final body composition and nutrient retention efficiency are shown in Table 6. No significant differences were found for dry matter, protein, and ash between the treatments ($p > 0.05$). The fat was significantly lower in the POU diet (9.39%) compared to the CONT diet (14%) ($p < 0.05$).

Table 6. Body composition (% wet weight) and protein efficiency retention of gilthead seabream fed the experimental diets.

	Initial	CONT	ORG	TRO	SBS	MIX	POU	SEM
Dry matter (%)	30.0	31.8	30.4	29.9	29.6	29	28.1	1.41
Protein (%)	17.0	16.7	16.7	16.7	16.5	16.7	17.5	0.33
Fat (%)	9.0	14.0^{a}	12.3^{ab}	11.8^{ab}	11.8^{ab}	10.5^{b}	9.4^{b}	0.42
Ash (%)	3.6	1.5	1.6	1.7	1.6	2.0	2.2	0.09
PPV (%)[1]		23.1^{a}	21.0^{a}	19.1^{ab}	19.9^{ab}	19.3^{ab}	16.1^{b}	0.43
PFV (%)[2]		54.2^{a}	41.6^{b}	37.1^{bc}	31.4^{bc}	26.7^{cd}	18.2^{d}	2.64

For each treatment, values are represented as mean ± standard error (n = 3). The values that do not share the same letter differ significantly (p < 0.05). Newman–Keuls test. 1 Productive protein value (PPV %) = protein retained (final fish protein × final biomass (g)) × 100 − initial fish protein x initial biomass (g)/protein ingested (kg ingested food ×% crude protein). 2 Productive fat value (PFV %) = fat retained (final fish fat × final biomass (g)) × 100 − initial fish fat × initial biomass (g)/fat ingested (kg ingested food × % crude fat).

Concerning productive protein value (PPV), there were no differences between the CONT, ORG, TRO, SBS, and MIX diets ($p > 0.05$). On the other hand, the fish fed the POU diet obtained the lowest value of PPV (16.11%) ($p < 0.05$). Regarding the EAA (Figure 2), it can be seen that almost all of the lowest values for the essential amino acids were obtained in the fish fed the POU diet ($p < 0.05$). The highest retention efficiency for phenylalanine, isoleucine, and leucine was observed in fish fed the SBS diet (16.56, 22.51, and 21.20%, respectively) ($p < 0.05$). The CONT diet showed the highest retention efficiency of Lys, Arg, Thr, and Val (29.74, 23.24, 19.21, and 20.05, respectively) ($p < 0.05$).

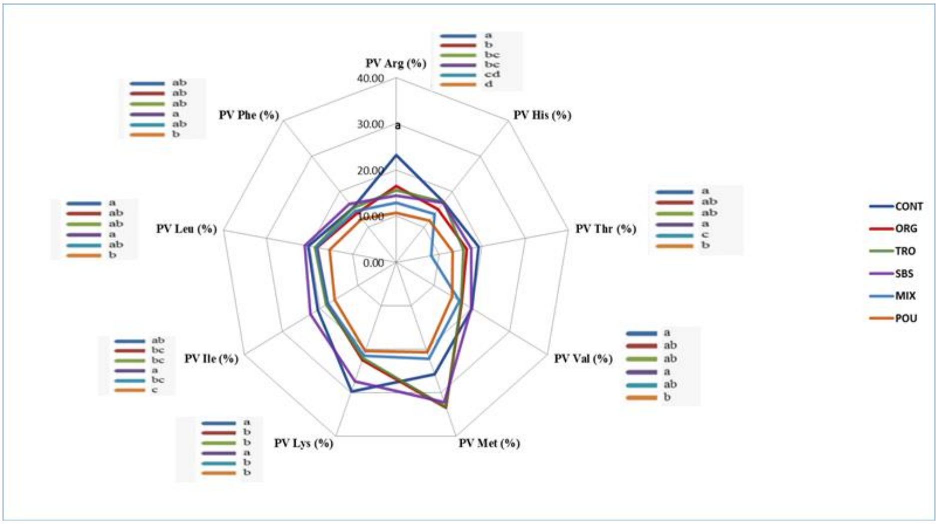

Figure 2. Productive value of essential amino acids (n = 3) for gilthead seabream fed experimental diets.

For each treatment, the values are represented as mean ± standard error (n = 3). The values that do not share the same letter differ significantly ($p < 0.05$). Newman–Keuls test. Productive values of amino acids (%) = (fish amino acid gain (g) × 100) /amino acid intake (g).

The results of the productive values of the primary fatty acids (g/100 g of wet weight) in the whole body of gilthead seabream fed different diets are shown in Table 7. The efficiency of retention of the fish fed the CONT diet was observed to have the highest values in most fatty acids ($p < 0.05$). Different organic ingredients in the diets showed significant changes in retention efficiency between treatments of several saturated fatty acids (SFAs) ($p < 0.05$). The retention efficiency of fish fed the POU diet was the lowest ($p > 0.05$). The retention efficiency of the monounsaturated fatty acids (MUFAs) showed significant differences ($p < 0.05$) between diets where the CONT diet obtained the highest value significantly (C16:1, C18:1n9t, and C18:1n9c), except C20:1 (Table 7). The retention efficiency of linoleic acid (C18:2n6c) and linolenic acid (18:3n3) was the highest ($p < 0.05$) in the fish fed the TRO diet (54.7 and 52.1, respectively). The lowest values ($p < 0.05$) were found for the POU diet. No significant differences (p > 0.05) were found for eicosapen-taenoic acid (EPA, 20:5n3) or docosahexaenoic acid (DHA, 22:6n3). Regarding the omega 3/omega 6 ratio, no significant differences ($p > 0.05$) were observed.

Table 7. Productive values of fatty acids in gilthead seabream fed the experimental diets (g/100 g of wet weight).

	CONT	ORG	TRO	SBS	MIX	POU	SEM
SFA [1]							
(C13:0)	12.3 ^{b}	18.5 ^{b}	67.6 ^{a}	12.8 ^{b}	14.0 ^{b}	9.7 ^{b}	3.49
(C14:0)	60.9	57.4	39.1	50.2	42.5	46.4	4.68
(C15:0)	53.4	54.4	38.6	39.7	46.5	35.2	5.56
(C16:0)	60.4 ^{a}	53.2 ^{ab}	50.0 ^{ab}	39.6 ^{bc}	40.5 ^{bc}	28.6 ^{c}	4.55
(C17:0)	68.6 ^{a}	64.7 ^{a}	44.6 ^{ab}	47.4 ^{ab}	46.7 ^{ab}	32.2 ^{b}	6.72
(C18:0)	60.6 ^{a}	46.0 ^{b}	40.0 ^{bc}	39.9 ^{bc}	36.8 ^{bc}	23.5 ^{c}	3.98
MUFA [2]							
(C16:1)	98.3 ^{a}	89.7 ^{a}	48.1 ^{b}	58.8 ^{b}	55.9 ^{b}	54.8 ^{b}	5.54
(C17:1)	87.3	92.3	56.7	67.9	66.2	56.9	18.15
(C18:1n9c)	95.6 ^{a}	70.1 ^{c}	86.9 ^{b}	95.7 ^{a}	73.9 ^{c}	73.6 ^{c}	9.46
(18:1n-7)	66.2 ^{a}	55.1 ^{ab}	45.4 ^{bc}	43.6 ^{bc}	43.9 ^{bc}	33.5 ^{c}	4.29
(C20:1)	57.3 ^{ab}	68.7 ^{a}	34.8 ^{b}	32.7 ^{b}	34.3 ^{b}	49.7 ^{ab}	6.38
PUFA [3]							
(C18:2n6c) LA [4]	43.8 ^{ab}	40.1 ^{ab}	54.7 ^{a}	39.1 ^{ab}	43.3 ^{ab}	29.3 ^{b}	3.95
(C18:3n3) LNA [5]	45.1 ^{ab}	34.8 ^{ab}	52.1 ^{a}	36.0 ^{ab}	37.1 ^{ab}	27.0 ^{b}	4.14
(C20:2)	84.5 ^{a}	68.8^{b}	38.5 ^{c}	39.8 ^{c}	30.7 ^{c}	22.6 ^{d}	4.48
(C20:3n6)	42.0 ^{a}	35.5 ^{ab}	30.2 ^{ab}	22.5 ^{b}	26.8 ^{ab}	28.5 ^{ab}	3.82
(C20:4n6) ARA [6]	47.8	48.7	37.4	43	46.2	37	6.84
(22:4n-6)	22	42.2	27.4	43.4	26.9	16.9	7.55
(22:5n-3)	98.3	95.1	70.1	71.5	61.8	67.8	10.58
(20:5n-3) EPA [7]	31.5	32	40	28.7	33.2	28.5	4.32
(22:6n-3) DHA [8]	53.5	53	49.2	45.9	50.4	48	9.07
ω-3/ω-6 ratio	1.6	1.4	1.3	1.4	1.1	1.6	0.16

For each treatment, values are represented as means (n = 3). The values that do not share the same letter differ significantly ($p < 0.05$). Productive values of fatty acids (%) = (fish fatty acid gain (g) × 100)/fatty acid intake (g). 1 SFA: saturated fatty acids. 2 MUFA: monounsaturated fatty acids. 3 PUFA: polyunsaturated fatty acids. 4 LA: linoleic acid. 5 LNA: linolenic acid. 6 ARA: arachidonic acid. 7 EPA: eicosapentaenoic acid. 8 DHA: docosahexaenoic acid.

4. Discussion

According to Craig and McLean, the need for certified protein sources significantly hinders the growth of organic aquaculture [29]. There is still much discussion about the certifiability of by-catch from commercial fisheries, byproducts, and processing wastes from aquaculture, fish, and meat processing industries as ingredients for organic aquafeed. The acceptability and availability of amino acids in these products are also questionable [30]. Vegetable protein sources pose challenges, especially for feeding higher-level carnivores such as seabream. They contain antinutritional factors and have low biological value due to essential amino acid deficiencies and poor digestibility [31]. Furthermore, including non-organic certified plant ingredients instead of fish ingredients in fish feeds also brings about the presence of undesirable substances [32]. Some commonly used pesticides in land-based agriculture have been identified in aquatic feeds. For instance, a recent extensive analysis of aquafeeds has revealed the potential presence of chlorpyrifos-methyl (CPM) [33]. A survey of commercially available aquatic feeds conducted in 2017 reported CPM levels ranging from 11 to 26 µg/kg [34]. On average, approximately 5–10% of the examined feed samples had CPM levels exceeding the detection limit.

This study observed the highest final weight in fish fed the CONT and ORG diets without significant differences ($p > 0.05$). In these diets, 30% of commercial fishmeal was used as a protein source. These high-quality fish meals are known to be the best protein source for fish thanks to their high digestibility and because their amino acid composition is very close to the need profile of most carnivorous aquaculture species [35,36]. Regarding the amino acid profile, in both the CONT and ORG diets, there is a greater quantity of essential amino acids compared to other diets, which also must impact the final growth results.

For the organic diets without a commercial fish meal, the TRO and SBS diets exhibited better growth compared to MIX and POU diets. However, previous studies of diets produced with the remains of the rest of the seabass and trout cannot be found. The growth results observed in the fish fed the TRO and SBS diets can be explained by the nature of the diet. Fish protein has an amino acid profile that closely matches the nutritional needs of the fish, which likely contributes to their improved growth compared to the control diet. The differences between the control diet and the TRO and SBS meal diets can be attributed to the fact that the raw materials used are the remains of these species.

The remains may affect factors such as protein availability or the processing of these raw materials, leading to variations in growth outcomes. Using trout meal and seabass meal byproducts promotes resource efficiency, waste reduction, and the establishment of a circular economy. One of the key advantages of using these byproducts is their positive environmental impact. Instead of discarding them, incorporating them into other products or processes minimises the need for additional resources and waste disposal. This approach fosters a more sustainable production cycle and contributes to environmental conservation [37]. There is currently no commercial organic supply chain for trout and seabass. These certified organic meal products should be manufactured in dedicated organic meal factories. The current availability of organic seabass and trout is insufficient to justify these factories' existence. However, if such products were established, it could greatly enhance the profitability of organic production.

It is worth noting that the fish from the MIX treatment, which included poultry meal in its composition, obtained a lower final weight than those containing organic ingredients and aquaculture proteins (TRO and SBS). The presence of poultry meal in the MIX treatment affected the growth of the gilthead seabream and may have impacted protein availability. Moreover, the lowest final weight was obtained with the POU treatment.

According to Regulation (EU) 2018/848 [16], Part III, paragraph (e) of Section 3.1 regarding feeding aquaculture animals, it dictates that “growth factors and synthetic amino acids will not be used.” Consequently, using amino acids is not allowed commercially in organic aquaculture. Its application in diet formulation at the production level would not be feasible unless sustainable plant amino acids are used, such as vegetable methionine, in the present work, even though its efficiency is lower (at the time of the design of the experiment, when the commercial company that provided the diet-only had vegetable methionine).

Concerning the fatty acids in the diet, they do not seem to be the determining factor in the present study, given the amount of fish oil in this diet. Likewise, it depends on the percentage of inclusion in the diet and its quality, as mentioned above. In the present study, the feed intake was numerically higher in the organic diet groups than in the control group. However, the differences were not statistically significant.

The fact that the FI and FCR were higher in the organic diet groups may indicate that the organic diets' nutrients were unbalanced. Hence, the animals needed to increase their feed intake to compensate for deficient nutrients, such as essential amino acids. This may be the result of the origin of the raw materials. Regarding the FCR, the highest values and those statistically different from the rest of the treatments were registered in the fish fed the POU diet. Because of the above and in agreement with the study by Karapanagiotidis *et al*. [38], a 100% replacement of fishmeal for poultry meal significantly increased the FCR and reduced the efficiency of feed utilisation. However, if the ECR is observed, the ORG and POU diets resulted in a higher investment of money to produce fish. The higher price of the ORG diet and the high FCR of the POU diet causes this worsening of the ECR. On the other hand, the growth of the CONT, TRO, SBS, and MIX diets entails a similar ECR: the better FCR of the CONT diet is compensated by the lower prices of the organic diets made with byproducts. In addition, mortality was higher in the POU treatment than in other studies [38], where no difference in mortality was evident between the treatments. This was possibly a consequence of the lower appetite of these fish, providing justification for their worse growth.

Some studies on seabream and seabass have been published that compare conventional diets with organic diets, obtaining better growth in organic diets [21,39] since these diets were formulated with a higher percentage of fish meal (63 and 56%) than the conventional ones (50 and 20%).

Regarding body composition and nutrient retention efficiencies, the fat was significantly lower in the poultry treatment diets (POU and MIX) compared to the ORG diet, which differs from the study by Sabbagh *et al*. [40,41], where no differences were found. However, it agrees with the findings of other studies in which a higher inclusion of poultry meal led to a decrease in body fat [38], possibly due to the lower growth obtained with this diet.

The CONT and ORG treatments show higher percentages for protein retention efficiency (PPV) and fat retention efficiency (PFV). This means that they use a higher proportion of proteins and lipids in their diet for their growth, and consequently, more significant growth is manifested. On the other hand, the PPV was significantly lower in the POU treatment and was similar to the MIX treatment, which is related to the low growth of the fish fed these types of diets.

The essential amino acid profile in the diet can also explain differences in amino acid retention efficiency. Some authors [42,43] noticed that protein retention efficiency decreases with the intake. Consequently, it seems logical that the efficiency retention of a single amino acid could be influenced by the feed composition, increasing the efficiency when the composition is lower. Some of the increased efficiencies observed in Figure 2 could be explained by observing the TRO diet, which is a low amount of histidine (9.70 g/kg) but has the highest retention efficiency for this EAA (16.98%). The same trend is evident in the diet SBS with phenylalanine, where there is a low amount of this amino acid (18.90 g/kg), and the retention efficiency is the highest (16.56%). In general, many of the high retention efficiencies of gilthead seabream could be due to a lower amino acid content. The fact that EAAs with higher concentrations in the organic diets have lower retentions in fish suggests that the EAA profile needs to be well balanced. Instead of being used for protein synthesis, these excessive dietary EAAs were catabolised. This results in the lower retention of EAAs with high concentrations in the organic diets.

The retention efficiency of fatty acids in seabream fed experimental diets is directly related to the fatty acid profile in different diets, as has been seen in other species such as *Salmo salar* [44,45] or *Dicentrarchus labrax* [46]. Even though the dietary profile of fatty acids differs by the type of feeding of the fish, the results agree with the studies carried out by other authors, where saturated fatty acids are represented mainly by C16: 0 and C18: 0, and those monounsaturated by C: 18: 1n9 [47]. The literature reports that those species that include significant amounts of linoleic acid (C18: 2n-6) or linolenic acid (C18: 3n-3) in their diet present lower concentrations of the C18: 1n-9t and C18: 1n-9 acids in their tissues [48]. The variations of these acids in the analysed species are probably multiple factors, among them the feeding of the fish, a determining element for their composition [49,50].

It is essential not to ignore the effect of lipid composition on the fatty acid composition of fish fed organic feed. From the data in Tables 3 and 7, the retention efficiency of n-6 and n-3 of fish lipids is greatly affected by the n-6 and n-3 of dietary lipids. When the dietary ratio is very high in n-6 fatty acids, fish tend to alter the proportion of PUFAs incorporated in favour of n-3 fatty acids [48]. It is common to see changes in fatty acid profiles by substituting fishmeal for other lipid sources. However, there needs to be more information on the effects of changes in the retention efficiency of fatty acids.

A study carried out in *Seriola dumerili* [51] fed fish with high levels of substitution of fish oils for a mixture of vegetable oils; however, in this case, such high differences were not obtained in terms of efficiency and retention, since the differences in growth concerning the control feed were not so relevant. On the other hand, it can be stated that they are closely related to the productive values obtained for fat, which was significantly lower in fish fed POU, as well as the low retention of EPA and DHA, which, together with the lower levels of these fatty acids in the diet, could have been the trigger for mortality observed in this group.

The highest productive values of FA were observed in the fish fed the CONT diet. In these diets, commercial fish oil was used as a lipid source. These high-quality fish oils are known to be the best lipid source for fish thanks to their high digestibility and their fatty acid composition availability [52]. These results agree with other studies that show that the dietary fatty acid compositions reflect FA compositions in marine fish [53]. It is perceived that variations in the fatty acid profile of meals are primarily reflected in the fish composition [54]. The productive values of the FA of the gilthead seabream show that when an FA is at a lower dietary level, its retention efficiency will increase; the opposite occurs when there is a higher level of FA.

This study found that the best growth occurred in the two control diets containing 30% fishmeal, regardless of whether the rest of the ingredients were organic. Regarding the experimental diets, the fish fed the TRO diet showed the highest growth, followed by the SBS and MIX diets; finally, the POU diet showed the lowest growth. The fish fed the POU diet exhibited the highest mortality, while those fed the CONT, ORG, TRO, SBS, and MIX diets presented similar mortality rates. Regarding nutrient retention efficiency, different organic ingredients in the diets showed significant changes in the retention efficiency of several fatty acids between the treatments. However, no significant differences were found in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Using trout meal and seabass meal byproducts in production offers various benefits, including resource efficiency, waste reduction, and promoting a circular economy. Incorporating these byproducts into other products or processes minimises environmental impacts and conserves resources. The availability of these raw materials depends on factors related to fish farming, fisheries management, and market demand. Implementing sustainable practices and establishing collaborations within the industry is crucial for maintaining a reliable supply chain.

However, the specific growth outcomes can be influenced by factors such as the composition of the diets and the presence of certain raw materials such as poultry meal.

One of the main factors impeding organic production growth is the higher cost of organic feed. However, this does not have to be the case. The present study demonstrates that organic feed can be obtained at competitive prices by utilising byproducts from other organic farms. Based on economic indices, completely replacing fishmeal with more organic alternatives containing organic fish byproducts is a promising alternative to feeding farmed fish organically. Total replacement and some efficiency parameters appear to affect growth, but slightly enough to still be economically convenient. The findings provide insights into the potential benefits of using organic ingredients in aquaculture diets. Therefore, it is recommended to continue increasing the knowledge in this sector to mitigate the impact of extractive fishing and more aquaculture sustainability, as well as the experimental conclusions that can be drawn.

Acknowledgement

The first author was supported by a complete grant from the Ministry of Higher Education of the Arab Republic of Egypt and the research project had been developed with the collaboration of the Biodiversity Foundation (Spanish Ministry for Ecological Transition and the Demographic Challenge), through the Pleamar Program, co-financed by the European Maritime and Fisheries Fund (EMFF).

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Chapter 2.

New Organic Raw Materials for Gilthead Seabream (*Sparus aurata)* Feeding and the Effects on Growth, Nutritive Parameters, Digestibility, and Histology

New Organic Raw Materials for Gilthead Seabream (*Sparus aurata)* Feeding and the Effects on Growth, Nutritive Parameters, Digestibility, and Histology

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Fishes, 2023

Tefal, E.; Jauralde, I.; Tomás-Vidal, A.; Martínez-Llorens, S.; Peñaranda, D.S.; Jover-Cerdá, M. New Organic Raw Materials for Gilthead Seabream (*Sparus aurata*) Feeding and the Effects on Growth, Nutritive Parameters, Digestibility, and Histology. *Fishes* 2023, *8*, 330. https://doi.org/10.3390/fishes8060330.

Abstract

A 120-day experiment investigated the new organic raw materials for Gilthead seabream (*Sparus aurata)* feeding on growth, nutritional parameters, digestibility, and histology. An organic control diet (CON) and three diets with 100% organic raw materials, the rest of rainbow trout, visceral Iberian pig, and insects as a protein source (TRO, IBE, and INS) were tested. After the experiment, growth, nutritional parameters, digestibility, and histology were measured. The CON diet-fed seabream obtained the highest weight; there was no difference between the experimental diets. The crude protein content was the highest in seabream fed the TRO and INS diets. Crude fat was the highest value observed in the CON diet. High digestibility was observed in seabream-fed IBE and INS diets. Except for EAA methionine (Met), there were no static differences in retention efficiency. The diet with the highest hydrolysis rate was IBE. Diet differences were significant but had the typical healthy liver morphology of seabream. Seabream fed on the TRO and INS diets had shorter distal gut measurements. In conclusion, the full substitution of fishmeal with organic raw materials, including rainbow trout remains, Iberian pig viscera, and insects, offers several benefits in terms of digestibility, histology, and growth performance and may help improve sustainable and healthy aquaculture practices.

Keywords: gilthead seabream; organic diets; organic raw materials; organic fish; organic production; fishmeal substitution; organic aquaculture.

Introduction

Organic aquaculture is a rapidly growing sector that focuses on sustainability, animal welfare, and reducing environmental impact. Organic production involves a combination of traditional methods, modern technology, and scientific knowledge to protect the environment, support fair and equitable relationships, and improve quality of life. Organic fish farming aims to reduce antibiotics, pesticides, and synthetic fertilizers, among other things, and promote natural inputs [1]. In the 27 European Union (EU) countries, the organic production of European seabass and gilthead seabream in 2020 was 2750 tons, representing only 1.5% of the total production of these species [2]. The European Market Observatory for Fisheries and Aquaculture (EUMOFA) states that the primary obstacles to organic aquaculture include the general lack of growth, insufficient innovation, and increased costs, particularly regarding organic feed [2]. To be considered organic certified production, the feed must be produced from: byproducts of other organic aquaculture species; organic plant production; trimmings and byproducts; fishmeal; and fish oil from certified fisheries, according to EU Regulation 1380/2013.

The demand for certified organic feed ingredients for aquaculture is much higher than the supply worldwide, leading to elevated prices and high production costs [3]. Although revised EU regulations now allow fishmeal and fish oil derived from sustainable fisheries, other alternative feed ingredients with high levels of essential amino acids (EAA) and lipids are still required. According to Gambelli *et al*. [4], it is suggested that enhanced research in aquafeed is necessary to improve the competitiveness of organic fish farming and foster the future growth of organic aquaculture. As such, replacing fishmeal with alternative protein sources in organic aquaculture feeds is particularly interesting. Alternative protein sources can totally or partially replace fishmeal (FM) in aquaculture diets, reducing feed costs and improving sustainability [5].

With various degrees of success, the replacement of FM in aquaculture diets with various soy products, such as soybean meal, soy protein concentrate, and soy protein isolate, has been described [6,7]. Animal protein byproducts, such as poultry byproductsmeal (PBM), have been studied as a partial or total replacement for FM in aquaculture diets due to the increasing cost and limited availability of FM [8].

Aquaculture protein by- products are a cost-effective and sustainable alternative to traditional fishmeal-based feeds. In addition to reducing the environmental impact of fish processing, these by- products can also help reduce the pressure on wild fish stocks harvested for fishmeal production. A few studies have explored aquaculture protein byproducts in aquaculture feed [9–12]. Li *et al.* [12] evaluated using a mixture of shrimp hydrolysate and plant proteins in diets for largemouth bass. The results showed that the combination could replace up to 30% of the FM in the diet without negatively impacting growth performance [12]. Similarly, a study by Gunathilaka *et al.* [11] investigated the use of shrimp protein hydrolysate and krill meal in the diets of red seabream (*Pagrus major*). The results showed that incorporating shrimp protein hydrolysate into the red seabream diet can decrease FM usage by up to 20% [11]. Previous studies evaluated the use of fish protein hydrolysate (FPH) in the diets of Nile tilapia. The results showed that the use of 10% FPH gave the highest growth performance, feed utilization, and protein utilization [10].

Gilthead seabream (*Sparus aurata)* is a notable species of farmed fish in the Mediterranean countries, with an annual global production of approximately 282.1 thousand metric tons in European countries [13]. Despite implementing new nutritional strategies that have reduced the use of fishmeal in seabream diets, it is still necessary to minimize fishmeal levels to improve aquaculture sustainability, and certified organic production continues to be a testimonial. Research on organic farming of gilthead seabream is scarce, and most studies focus on growth performance, welfare, and quality aspects compared to conventional farming [14–16]. A study on an industrial scale found differences in growth performance, metabolic status, and fillet composition between organically produced fish and conventional ones, indicating the need for more research to improve organic feed formulation [17]. On the other hand, it was reported by Estevez *et al.* [3] that to evaluate new organic ingredients, such as green pea protein and brown seaweed, to replace fish meals in feeds suitable for organic production. No adverse effects were observed on fish growth, their muscles' composition, health, quality, or nutritional value [3].

Improving the sustainability of aquaculture through the organic substitution of fishmeal is an important goal that must be reached as soon as possible. Success in achieving this goal must ensure that such substitution does not harm the performance or efficiency of the growth of gilthead seabream.

Materials and Methods

Ethics Approval

Following Royal Decree 53/2013 and the European Directive 2010/63/EU on the protection of animals used for scientific research, 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) to minimize the suffering of animals.

Experimental Conditions

Four different diets were fed to the seabreams for 120 days. In total, 300 fish with an average mean weight of ~93 ± 3.82 g and an average mean length of 16 ± 1.6 were used and were distributed in 12 concrete tanks of 4000 L each with octagonal-shaped tanks. The experiment was carried out on the Animal Science Department of the Polytechnic University of Valencia (UPV) farm from August to November 2021, after ten days of adaptation for the seabream, which weighed an average of ~93 ± 3.82 g.

The trial was within a saltwater recirculating system of 75 m^3 of capacity with a rotary mechanical filter and a 6 m^3 capacity gravity biofilter, with 25 animals in each tank. Feeding was done manually until apparent satiety, twice daily, at 09:00 and 16:00, six days a week, from Monday to Saturday. Additionally, throughout the experiment, the physical and chemical parameters of the tanks were monitored: dissolved oxygen 8.7 ± 1.6 mg L^{-1}, temperature 21 ± 1 °C, ammonium level 0.22 mg L^{-1}, nitrates 104.2 mg L^{-1}, nitrites 0.38 mg L^{-1}, salinity 33.3 ± 2.4 g L^{-1}, and pH 7.49. The photoperiod was natural (~12.5 h) (August to November 2021), and the lighting in each tank was the same.

Experimental Diets

The diets were manufactured at the Universitat Politècnica de València (UPV) facilities using a Clextral BC45 semi-industrial extruder using the cooking-extrusion process (CLEXTRAL BC-45, St. Etienne, France).

Four diets were tested in triplicate: the CON diet, with FM as a protein source; the TRO diet, with FM coming from organic trout remains after processing; the INS diet, which uses organic insect meal instead of FM; and the IBE diet, which contains organic Iberian pig viscera to replace FM (Table 1). The replacement of fishmeal was 100% in all experimental diets.

The following processing parameters were used: a screw speed of 100 rpm, a pressure range of 40–50 atm, and a temperature of 110 °C. Organic raw materials formulated the four extruded diets (Table 1). All the organic ingredients come from organically certified producers with the EU organic label. Trout was self-processed to extract the meat slices, and just the remaining parts were carefully cut, oven-dried, and ground into a suitable form for use in the feed. The remaining parts used for the Iberian pig meal were the liver, intestines, and heart, which were cut into small pieces. After oven drying, they were ground into a suitable form for incorporation into the feed. The insect component, larval insects, was utilized. They were already dried and then ground for inclusion in the diets. The level of inclusion of the TRO, IBE, and INS was sufficient to replace fishmeal and maintain the total protein constant. The diets were supplemented with sustainable supplies of vegetable methionine and calcium phosphate. Dietary formulation and processing using organic raw components labeled and approved following Regulation (EU) 2018/848 [18]. Once completed, they were packaged and stored in a commercial refrigerator at 4 °C.

Table 1. Ingredients and nutritional composition of the experimental diet.

Ingredients (g Kg $^{-1}$)	**CON**	**TRO**	**INS**	**IBE**
Raw materials (g kg^{-1})				
Fishmeal [a]	300			
Organic rest of rainbow trout [b]		400		
Organic insect meal [c]			390	
Organic Iberian pig viscera [d]				345
Organic wheat [e]	206	168	73	230
Organic wheat gluten [f]	110	110	198	110
Organic soybean meal [g]	228	251	250	220
Fish oil [h]	20	25	25	25
Organic soybean oil [i]	100	26	19	25
Calcium phosphate [j]	23		25	25
Vegetable methionine [k]	3	10	10	10
Vitamins [l]	10	10	10	10

Nutritional composition				
Dry matter	88.5	93.0	88.9	85.5
Crude Protein	43.0	43.1	44.2	44.0
Crude lipid	18.6	20.3	18.5	20.6
Ash	7.4	9.7	8.1	5.7
Calculated Gross Energy (kJ/g) [m]	20.6	21.5	20	20

CON: control; TRO: trout; IBE: Iberian; and INS: insect. [a] Fishmeal (91.9% DM, 73.9% CP, 11.2% CL, 14.3% Ash); CORPESCA SA. [b] Rest of rainbow trout (98.60% DM, 64.60% CP, 21.7% CL, 12.40% Ash). (Naturix, Valderrebollo, Guadalajara, Spain). [c] Insect meal (92.6% DM, 37.6% CP, 28.5% CL, 20% CHO, 13.9% Ash). [d] Visceral Iberian pig (92.6% DM, 53.0% CP, 28.6% CL, 14.6% CHO, 3.8% Ash). [e] Organic wheat (92.3% DM, 12.7% CP, 1.3% CL, 1.7% Ash, 19.7 kJ^{-1} energy); (PIENSOS ecoLUCAT, Barrax, Albacete, Spain). [f] Organic wheat gluten. [g] Organic soybean meal (94.6% DM, 43.1% CP, 9.3% CL, 6.3% Ash, 19.7 kJ^{-1} energy); (PIENSOS ecoLUCAT, Barrax, Albacete, Spain). [h] Fish oil (Industrias Afines, L.L. (Arpo), Polgono industrial A Veigadaa, Ra as Baloutas, de Abaixo, 24, 36416, Pontevedra). [i] Organic soybean oil (Clearspring Ltd., Acton Park Estate, London W3 7QE, United Kingdom). [j] Calcium phosphate. [k] Vegetable methionine (Adibio S.L. | Edificio Galileo, C/Enebros 74, 2ª planta | 44002 Teruel (Espaa). [l] Mix of vitamins and minerals (g kg^{-1}): Premix: 25; Choline, 10; DL-a-tocopherol, 5; ascorbic acid, 5; (PO4)2Ca3, 5. Premix composition: retinol acetate, 1,000,000 IUkg^{-1}; calciferol, 500 IUkg^{-1}; DL-a-tocopherol, 10; menadione sodium bisulfite menadione, 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. [m] Calculated Gross Energy (kJ/g) = [51.8 × (%C/100)) − (19.4 × (%N/100)].

Growth Control

The fish were monitored daily in tanks and weighed every 30 days while anesthetized with clove oil containing 87% eugenol (Guinama®, Valencia, Spain) in 150 mg L^{-1} of water. This was done to assess fish growth throughout the experiment, establish growth parameters, and determine the health of the fish. The fish were not fed the day before being weighed. Five fish were sampled at the beginning of the experiment and kept at −30 °C for further analysis of total body composition. At the end of the study, ten animals in each tank were sampled to evaluate biometric parameters. Three fish from each tank (to ensure we have representative samples for analysis) were randomly selected for sampling and pooling to determine the approximate composition and amino acids.

Analysis of Nutritional Composition and Amino Acids

The whole body of the fish and the composition of the diets were examined using the procedures outlined in AOAC (Association of Official Analytical Chemists) [19]: dry matter (105 °C to constant weight), ash (incinerated at 550 °C for five hours), crude protein (determined using the direct combustion method DUMAS using LECO CN628, Geleen, Netherlands), and crude lipid. Diets are extracted with methyl ether using the ANKOMXT10 extractor (Macedon, NY, USA). Each analysis was carried out three times. The procedure previously described by Bosch *et al*. [20] was used to analyze the AA of diets and body fish. A Waters HPLC system (Waters 474, Waters, Milford, MA, USA) consisting of two pumps (Model 515, Waters), an autosampler (Model 717, Waters), a fluorescence detector (Model 474, Waters), and a temperature control module was used.

Aminobutyric acid was first introduced as a standard internal pattern prior to hydrolysis. The AA was derivatized using AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Methionine and cysteine were recognized as methionine sulphone and cystic acid, respectively, following oxidation with performic acid. After being separated with a reverse-phase C-18 column by Waters Acc, the tag AA was changed to methionine and cystine (150 mm, 3.9 mm). The EAA content of the experimental diets is indicated in Table 2. Amino acid tests were performed in duplicate on every sample.

Table 2. Composition of essential and non-essential amino acids in experimental diets.

Diets	CON	TRO	INS	IBE
Essential amino acids (g 100 g^{-1}) (EAA)				
Arginine	2.94	2.81	2.59	2.42
Histidine	2.26	0.91	1.26	1.13
Isoleucine	1.51	1.63	1.80	1.70
Leucine	3.00	3.04	3.08	3.12
Lysine	2.32	2.39	2.02	1.97
Methionine	1.08	1.01	0.77	1.15
Phenylalanine	1.84	1.78	2.12	2.06
Threonine	2.28	1.50	1.51	1.46
Valine	2.02	2.08	2.41	2.35
Non-essential amino acids (g 100 g^{-1}) (NEAA)				

Alanine	2.07	2.11	2.14	2.06
Aspartic acid	3.57	3.53	3.88	3.73
Cysteine	0.51	0.48	0.56	0.52
Glutamic acid	9.74	9.11	9.96	10.24
Glycine	2.62	3.06	2.46	2.25
Proline	2.78	2.87	3.51	3.24
Serine	2.10	1.97	2.25	2.22
Tyrosine	1.34	1.33	1.94	1.66

CON: control; TRO: trout; IBE: Iberian; and INS: insect.

Indices of Growth and Biometric Parameters

With the sampling carried out, it was possible to obtain data on the weight of the individuals, the number of fish in each tank, and their total biomass. Growth and nutrient efficiency indices, such as final weight (FW), specific growth rate (SGR), survival (S), feed intake (FI), and feed conversion ratio (FCR), were calculated at the end of the study, considering the tank as an experimental unit. Ten fish were randomly selected from each tank to obtain the biometric parameters. The fish were anesthetized with clove oil containing 87% eugenol (Guinama®, Valencia, Spain) in 150 mg/L^{-1} of water. Total length (cm), total weight (g), liver weight (g), carcass weight (g), and visceral fat weight (g) were measured to obtain the visceromatic index (VSI), hepatosomatic index (HSI), visceral fat index (VFI), and condition factor (CF).

Digestibility Assay

After the growth experiment, another experiment was conducted in another system prepared to estimate the digestibility of different diets for gilthead seabream. The study was conducted at the Animal Science Department of the Polytechnic University of Valencia (UPV) farm using a semi-closed marine recirculating system with 190 L fiberglass tanks; each trial lasted 30 days. Faecal material was collected in a settling column, dried in an oven, and analyzed for its nutritional content and inert markers. Chromic oxide (5 g kg^{-1}) was used as an indigestible marker to assess the apparent digestibility of the diets. Dry matter, crude protein, energy, calcium, and phosphorus were also analyzed using the same methods. The apparent digestibility coefficients (ADCs) were calculated for each diet, and the results were compared to determine the most digestible diet [21]. The following formulas were used to estimate the ADC of diets for dry matter (ADC*dm*, %):

$ADCdm\ \% = 1 - (\%\ Cr_2O_3 \text{ in diet}/\%\ Cr_2O_3 \text{ in feces})$. (1)

The following equations determined the percentage ADCs of every dietary nutrient, including calcium, phosphorus, protein, and energy:

$ADCnut = 1 - (\text{marker diet}/\text{marker feces}) \times (\text{nutrient feces}/\text{nutrients})$ (2)

In this equation, the variables “nutrient diet (g/kg)” and “nutrient feces (g/kg)” represent the relevant nutritional components of the diet and feces, such as protein or energy. Marker levels of the diet and feces are indicated by the measurements “marker diet” (g/kg) and “marker feces” (g/kg), respectively.

In Vitro Hydrolysis Assay

Conditions that simulated the digestive tract of juvenile gilthead seabream were used to conduct an in vitro hydrolysis test [22]. Six hours after feeding, samples of seven juvenile gilthead seabream were taken, each weighing an average of 256 g, to confirm the presence of digestive and intestinal-related enzymes. The fish were euthanized by immersing them in ice-cold water with a small amount of clove oil as an anesthetic. They were then promptly dissected to obtain the digestive tract. The digestive tract was divided into two sections: 1- the proximal intestine, which included the diffuse pancreas and the pyloric caecum; and 2- the stomach. The tissues were used to produce the extracts to measure proteases and amylase activities following the conditions shown in Table 3.

Table 3. Conditions were carried out in the protein hydrolysis assay.

	Acid Stage	Alkaline Stage
E:S ratio (U/mg protein) *	4.0	8.5
PH	3.5	8.5
Time (hours)	1.5	3.5
Temperature (°C)	25	25

***** E:S ratio: enzyme/substrate ratio.

An assay was carried out for each diet in triplicate plus a blank. The blank was constructed by inactivating the enzymatic extracts with heat before their inclusion in the bioreactors. It allowed quantifying the amount of amino acids in the extracts and diet. That is released by solubilization and not by enzymatic hydrolysis.

Liver and Intestinal Histology

After the growth experiment, the liver and distal intestine (DI) were dissected from the guts of three fish; each tank was fed experimental diets. Samples were kept in formalin buffered with phosphate (4%, pH 7.4). Following standard histological practices, all formalin-fixed tissues were systematically dehydrated in ethanol, equilibrated under ultra-clean conditions, and embedded in paraffin. Transverse sections of each paraffin block were cut using a Shandon hyper-cut microtome and then stained with hematoxylin and eosin for analysis. An Eclipse E400 Nikon light microscope from Izasa S.A. in Barcelona, Spain, was used to analyze 100 sections of the distal intestine and 400 sections of the liver. Hepatocytes and their nuclei were examined and their diameters measured to evaluate the effect of diet on the liver. We evaluated villus length (VL), villus thickness (VT), lamina propria (LP), muscle layer (ML), submucous layer (SML), and serous layer (SL).

Statistical Analysis

Using the Statgraphics® Plus 5.1 statistical program (Statistical Graphics Corp., Rockville, MO, USA), the results of various growth and nutrient indices, biometric indices, retention of AA, ADC, in vitro hydrolysis, and histological measurements were analyzed using an analysis of variance with a *Newman-Keuls* test for the comparison of means. Initial covariate weights were used for the study of growth indices. The findings are represented as the mean± standard error (SEM). The significance level was established at $p < 0.05$.

Results

Growth and Nutritional Parameters

The final weight of fish (FW), the specific growth rate (SGR), the survival, feed intake (FI), and the FCR of gilthead seabream fed experimental diets are shown in Table 4. The initial weight of the gilthead seabream ranged from 89.1 to 96.5 g. At the end of the experiment, the CON diet reached a higher FW (328.4 g), whereas the rest of the diets reached a similar FW, around 250 g. The rest of the parameters did not present significant differences. The survival rate of seabass ranged from 78.6% to 91.9%, and FI ranged from 1.65 to 2.0 g 100 g^{-1} fish day^{-1}.

Table 4. Growth and nutritional parameters of gilthead seabream fed experimental organic diets.

Diets	CON	TRO	INS	IBE	SEM	*p* Value
Initial weight (g)	90.3	89.1	96.5	96.0	1.34	0.1329
Final weight (g)	328.4 ^{a}	263.7 ^{b}	270.8 ^{b}	244.7 ^{b}	12.04	0.0290
Survival (%)	89.8	78.6	91.9	81.0	7.87	0.6013
SGR (% day^{-1}) [1]	1.15	0.95	0.9	0.85	0.04	0.4687
FI (g 100 g $fish^{-1}$ day^{-1}) [2]	1.7	2.0	1.65	1.85	0.15	0.4593
FCR [3]	2.05	1.9	2.25	1.7	0.31	0.6574

CON: control; TRO: trout; IBE: Iberian; and INS: insect. Values are represented as mean ± stander error (n = 3) for each treatment. Values that do not share the same letter differ significantly ($p < 0.05$). [1] Specific growth rate (SGR) = 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 consumption (g)/weight gain (g).

Body Composition, Retention Efficiency, and Biometric Indices

The nutritional composition of the whole body and the retention efficiency of protein and fat are shown in Table 5. The results show that the initial dry matter was 31.3, which increased after feeding with all experimental diets. The protein content decreased in all experimental groups. The protein content was the highest in gilthead seabream fed the TRO and INS diets (52.5 and 50.6%, respectively) and the lowest in the CON diet (47.2%). The fat content increased in all experimental diets, with the highest value observed in the CON diet (43.7%). No differences were found in the ash content.

Protein and fat retention efficiencies of gilthead seabream for protein and fat were also measured. Productive protein value (PPV) did not present a difference for the different diets, but productive fat value (PFV) was statistically higher for the CON and INS diets.

Table 5. Body composition in dry matter and retention efficiencies of gilthead seabream at the beginning and after feeding with experimental diets (%)

	Initial	CON	TRO	INS	IBE	SEM	*p* Value

Dry matter	31.3	34.7	35.5	36.6	35.2	1.00	0.2532
Protein	57.9	47.2 ^{b}	52.5 ^{a}	50.6 ^{a}	49.6 ^{ab}	0.80	0.0401
Fat	26.4	43.7 ^{a}	37.1 ^{b}	40.3 ^{ab}	38.7 ^{b}	1.06	0.0002
Ash	10.1	9.1	7.7	7.5	10.3	0.96	0.3551
PPV [1]		29.3	19.0	22.6	19.3	2.74	0.1507
PFV [2]		62.6 ^{a}	32.9 ^{b}	52.6 ^{a}	36.2 ^{b}	3.83	0.0121

CON: control; TRO: trout; IBE: Iberian; and INS: insect. Values are represented as mean ± standard error (*n* = 3) for each treatment. Values that do not share the same letter differ significantly ($p < 0.05$). [1] Productive protein value (PPV%) = protein retained (final fish protein × final biomass (g)) × 100—initial fish protein × initial biomass (g)/protein ingested (kg of food ingested food × % crude protein). [2] Productive fat value (PFV%) = fat retained (final fish fat × final biomass (g)) × 100—initial fish fat × initial biomass (g)/fat ingested (kg of food ingested food ×% crude fat).

Regarding biometric parameters in Table 6, no differences were observed in VSI. HSI had the lowest value in the TRO group (1.4) and the highest in the CON and INS groups (2.1 and 2.0, respectively). The CF was highest in the CON diet (2.2) and lowest in the IBE diet (1.89). The VFI was high in the IBE diet (1.9).

Table 6. Biometric indices at the end of the experiment.

Diets	**CON**	**TRO**	**INS**	**IBE**	**SEM**	***p* Value**
VSI [1] (%)	7.5	8.1	6.6	7.7	0.54	0.2879
HIS [2] (%)	2.1 ^{a}	1.4 ^{b}	2.0 ^{a}	1.7 ^{ab}	0.14	0.0014
CF [3] (g/cm^{3})	2.2 ^{a}	1.9 ^{ab}	2.0 ^{ab}	1.8 ^{b}	0.10	0.0235
VFI [4] (%)	1.5 ^{ab}	1.1 ^{ab}	0.86 ^{b}	1.9 ^{a}	0.25	0.0262

CON: control; TRO: trout; IBE: Iberian; and INS: insect. Values are represented as mean ± standard error (n = 9) for each treatment. Values that do not share the same letter differ significantly ($p < 0.05$). [1] Visceral index (VSI) (%) = (visceral weight (g)/total weight of fish. (g)) × 100. [2] Hepatosomatic index (HSI) (%) = (liver weight (g)/total fish weight (g)) × 100. [3] Condition factor (CF) (g/cm3) = (total weight of fish (g)/length3 (cm)) × 100. [4] Visceral fat index (VFI) (%) = (visceral fat weight (g)/total fish weight (g)) × 100.

Digestibility

The results indicate the ADC values of varied diets for dry matter, calcium, phosphorus, gross protein, and gross energy (Table 7). For dry matter, the highest ADC value was observed in the IBE diet (85.8%), followed by INS (83.0%) and TRO (74.7%), while the lowest value was recorded in the CON diet (63.8%). Calcium ADCs were the highest in the INS diet (55.5%), followed by the TRO and CON diets (51.5% and 37.8%, respectively), while the lowest value was recorded in the IBE diet (35.0%).

For phosphorus, the ADC values ranged from 69.8% to 73.2%, with no significant differences between diets. For gross protein, the highest ADC value was observed in the INS diet (93.0%), followed by IBE (91.7%), TRO (88.5%), and CON (84.2%). Gross energy ADCs were the highest in the INS and IBE diets (90.2% and 90.0%, respectively), followed by TRO (84.6%) and CON (78.4%).

Table 7. Apparent digestibility coefficients of dry matter and different nutrients of gilthead seabream fed experimental diets

ADC (%) *	CON	TRO	INS	IBE	SEM	*p* Value
Dry matter	63.8 ^{b}	74.7 ^{ab}	83.0 ^{a}	85.8 ^{a}	4.19	0.0114
Calcium	37.8 ^{ab}	51.5 ^{ab}	55.5 ^{a}	35.0 ^{b}	3.32	0.0057
Phosphorus	63.2	69.8	73.2	73.1	3.97	0.2669
Gross protein	84.2 ^{c}	88.5 ^{b}	93.0 ^{a}	91.7 ^{a}	0.87	0.0000
Gross energy	78.4 ^{c}	84.6 ^{b}	90.2 ^{a}	90.0 ^{a}	1.22	0.0000

CON: control; TRO: trout; IBE: Iberian; and INS: insect. Values are represented as mean ± standard error (n = 3) for each treatment. Values that do not share the same letter differ significantly ($p < 0.05$). * Apparent digestibility coefficients (ADC). ADCdm = 100 − (100 × (% Cr2O3 in diet/% Cr2O3 in feces)). ADCnut = 100 − (100 × (% feed marker/% feces marker) × (% nutrient. energy. amino acid. or fatty acid in urine/% of nutrient. energy. amino acid. or fatty acid in feed)).

Retention Efficiency of Essential Amino Acids

Table 8 shows the ability of the fish to retain EAA. Except for EAA methionine (Met), there were no static differences in the retention efficiency of EAA in gilthead seabream- fed experimental diets. The retention efficiency of Met was highest in gilthead seabream fed the INS diet (36.8%), significantly higher than the diets of CON, TRO, and IBE (26.1%, 21.5%, and 20.3%, respectively).

Table 8. Retention efficiency of essential amino acids from gilthead seabream-fed experimental diets (%).

Diet	CON	TRO	INS	IBE	SEM	*p* Value
Arginine	32.30	25.15	27.55	21.75	4.82	0.5354
Histidine	36.25	31.95	22.05	17.85	7.62	0.4019
Isoleucine	34.40	24.35	24.05	23.95	3.36	0.2140
Leucine	31.70	23.30	23.90	20.20	3.29	0.2297
Lysine	44.20	30.05	40.55	34.95	4.01	0.2082
Methionine	26.1 a^{b}	21.5 ^{b}	36.8 ^{a}	20.3 ^{b}	3.19	0.0554
Phenylalanine	27.50	19.65	18.95	16.25	2.67	0.1403

Threonine	26.80	27.45	26.50	20.50	6.13	0.8391
Valine	31.70	22.95	21.70	17.50	3.48	0.1623

CON: control; TRO: trout; IBE: Iberian; and INS: insect. Values are represented as mean ± standard error (n = 3). For each treatment. Values that do not share the same letter differ significantly ($p < 0.05$).

In Vitro Hydrolysis Assay

The results depicted in Figure 1. demonstrate the release of amino acids through the membrane following protein hydrolysis in experimental diets. Notably, the hydrolysis values were found to be remarkably similar across all four diets that were evaluated.

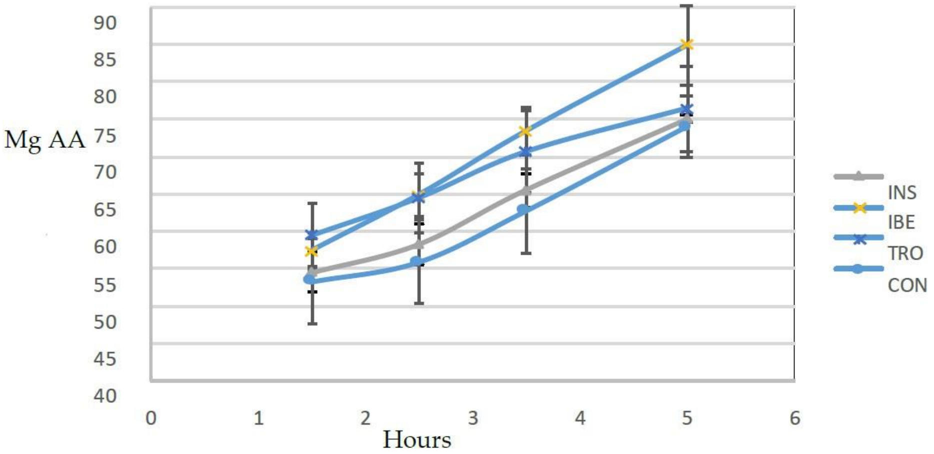

Figure 1. Release results of amino acids that cross the membrane after hydrolyzing from the protein of all experimental diets.
CON: control; TRO: trout; IBE: Iberian; and INS: insect.

The study investigated the dynamics of protein hydrolysis in experimental diets, and the findings, including linear equations and corresponding time points, are presented in Figure 2. The linear equations illustrate the relationship between the degree of protein hydrolysis (y) and the time elapsed for each diet (x). The slope of the equation reflects the rate of protein hydrolysis, while the intercept indicates the initial degree of hydrolysis at time zero. Notably, the rate of protein hydrolysis varied among the experimental diets.

The diet exhibiting the highest hydrolysis rate was IBE, with a slope value of 7.91. CON and INS followed closely, with slopes of 6.051 and 6.02, respectively. On the other hand, the diet with the lowest hydrolysis rate was

TRO, with a slope of 4.87. Furthermore, the time points at which the degree of hydrolysis was measured differed across the diets. The IBE diet had the shortest time of 6.91 h, while the TRO diet had the longest time of 9.75 h. These results highlight the variations in protein hydrolysis rates and the different time frames required for achieving specific degrees of hydrolysis among the experimental diets.

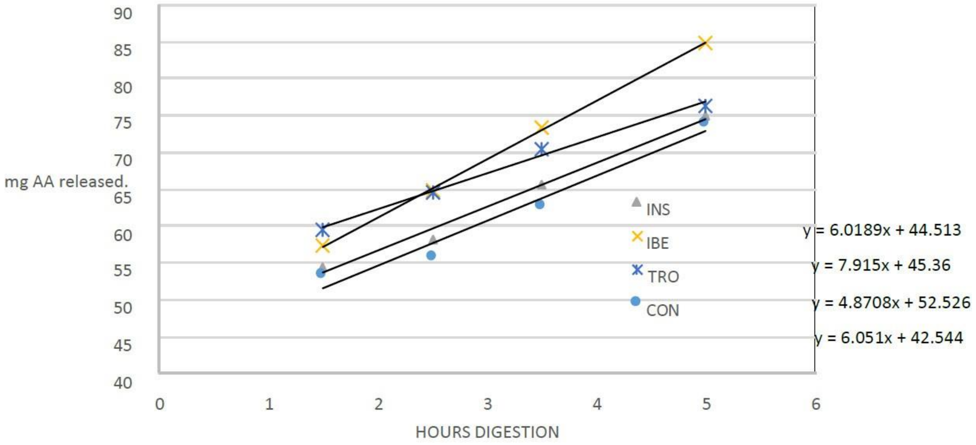

Figure 2. Equations of linear adjustments to the protein hydrolysis dynamics of all experimental diets

CON: control; TRO: trout; IBE: Iberian; and INS: insect.

Histology of the liver and intestinal

The liver histology results (Table 9) show that the nucleus and diameter of the hepatocytes varied between experimental diets. Differences between diets were statistically significant. The diet with the largest nucleus diameter was CON, with a mean value of 4.72 µm, followed by the IBE diet, with a mean value of 3.50 µm, while the diet with the minor nucleus diameter was TRO, with a mean value of 3.19 µm. Similarly, the diet with the largest hepatocyte diameter was CON, with a mean value of 12.46 µm, followed by the IBE diet with a mean value of 9.73 µm. In contrast, diets with a small diameter of hepatocytes were TRO and INS, with a mean value of 8.48 and 8.52 µm, respectively.

Table 9. Histological measures of the liver of gilthead seabream fed experimental diets

Diets	CON	TRO	INS	IBE	SEM	*p* Value

Nuclei diameter (µm)	4.72 ^{a}	3.19 ^{c}	3.28 ^{bc}	3.51 ^{b}	0.09	0.0021
Hepatocyte diameter (µm)	12.46 ^{a}	8.48 ^{c}	8.52 ^{c}	9.73 ^{b}	0.20	0.0000

CON: control; TRO: trout; IBE: Iberian; and INS: insect. Different letters indicate significant statistical differences ($p < 0.05$)—Newman-Keuls test. Values are the mean ± SEM (standard error of the mean) ($n = 100$).

Regarding the liver histology study of each treatment (Figure 3), small, slightly granular nuclei with homogeneous morphology were observed.

There was hardly any accumulation of lipids or displacement of the nucleus due to the vacuoles, presenting the typical morphology of a healthy gilthead seabream in all the treatments.

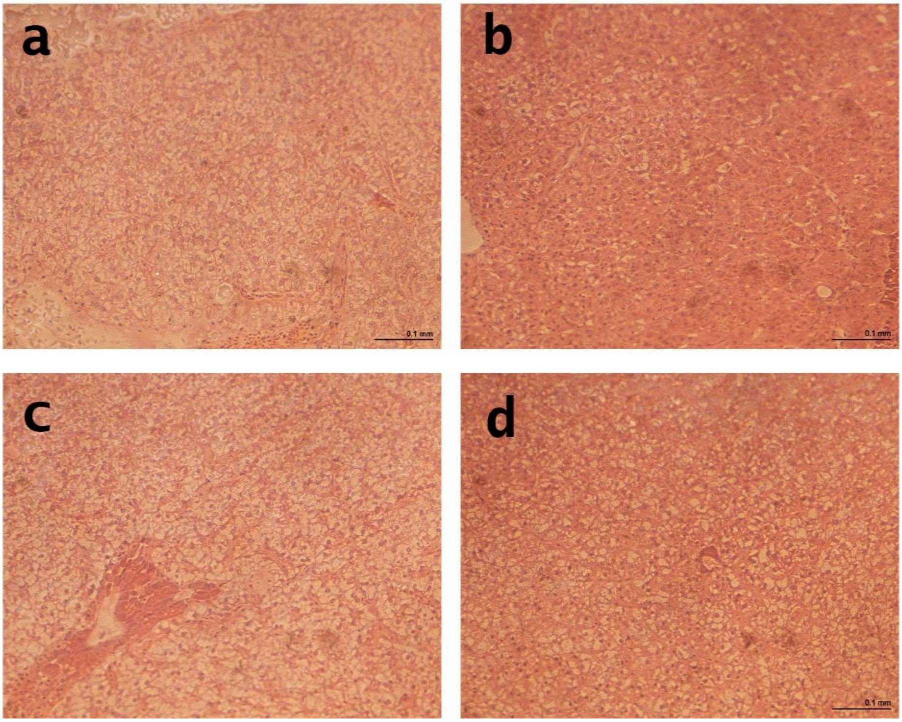

Figure 3. Histological detail of the liver (20×) of the gilthead seabream fed the experimental diet.

CON (a): control; TRO: trout; (d) IBE (b): Iberian; and INS (c): insect. Hematoxylin-Eosin staining.

Table 10 shows the results of the distal gut measurements. The results show that the experimental diets had differential effects on the distal measures of the intestinal tract in gilthead seabream. The differences between diets were statistically significant. The diet with the highest SL was CON, with a value of 106 µm, while the diet with the smallest SL was TRO, with a value of 53 µm. Similarly, the diet with the highest SML and VL was CON, while the diet with the lowest values for these measurements was TRO and IBE. However, the diet with the most significant ML, VT, and LP was CON IBE, with mean values of 146 µm, 218, and 63 µm, respectively, while the diet with the smallest values for these measurements was TRO and INS.

Table 10. Effect of the different diets on distal measurements of the gut in gilthead seabream

	CON	TRO	INS	IBE	SEM	*p* Value
SL (µm)	106 ^{a}	53 ^{b}	69 ^{b}	95 ^{a}	4.5	0.0182
ML (µm)	146 ^{a}	63 ^{c}	74 ^{c}	100 ^{b}	6.6	0.0124
SML (µm)	65 ^{a}	36 ^{b}	39 ^{b}	66 ^{a}	3.7	0.0257
VL (µm)	1336 ^{a}	741 ^{b}	741 ^{b}	1502^{a}	81.7	0.0110
VT (µm)	218 ^{a}	91 ^{c}	92 ^{c}	174 ^{b}	6.7	0.0000
LP (µm)	63 ^{a}	24 ^{c}	24 ^{c}	49 ^{b}	2.8	0.0001

CON: control; TRO: trout; IBE: Iberian; and INS: insect. Equal letters in the same row do not indicate significant differences between the means ($p < 0.05$)—Newman-Keuls test. Values are the mean ± SEM (standard error of the mean) ($n = 20$). SL: serous layer. ML: muscular layer. SML: submucous layer. VL: villi length. VT: villi thickness. LP: lamina propria.

Among diets, there was a similar morphology typical of the intestine of gilthead seabream. No mucosal alterations or highly vacuolated enterocytes were observed, and all treatments had goblet cells, mainly at the base of the villi, another indication of healthy intestines (Figure 4).

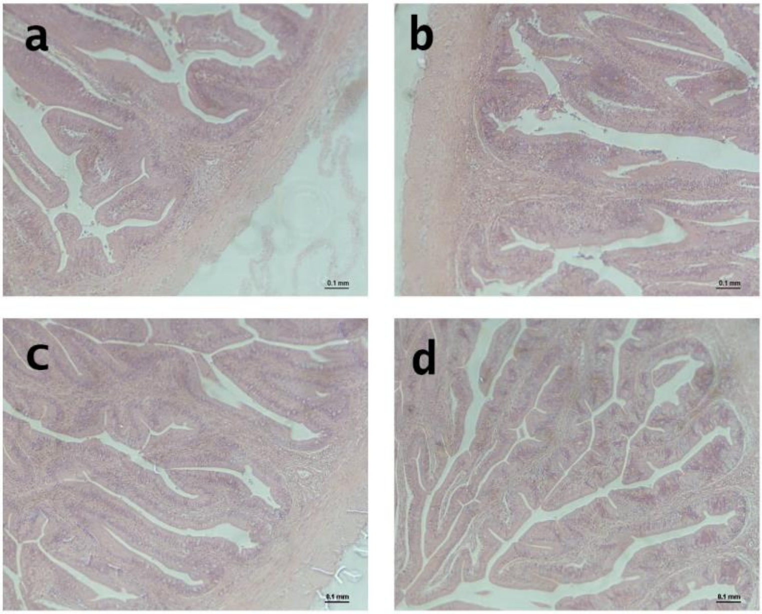

Figure 4. Histological detail of the hindgut (10×) of gilthead seabream fed experimental diets.

CON (a): control; TRO: trout; (d) IBE (b): Iberian; and INS (c): insect. Hematoxylin-Eosin staining**.**

Discussion

Improving organic fish's growth and value is a critical research area, particularly in developing suitable feeds for organic farming. However, the need for appropriate raw materials ideal for creating well-balanced diets and EU regulations have been significant obstacles to producing organic carnivorous fish feeds, which have limited the growth of organic aquaculture production [23]. The focus on feed formulation for organic production has shifted to the search for alternative raw materials that can serve as good protein sources due to the scarcity of fishmeal. This has become crucial in meeting the growing demand for protein sources [24]. However, the optimal ratio of marine and plant- origin proteins [25], marine and animal byproducts, and insects in organic aquaculture feed has yet to be established.

Furthermore, while organic marine and animal byproducts and insects can be incorporated into feed to some extent, the effect on growth, feed utilization, digestibility, retention efficiency, and histology must also be considered. Organic diets can substitute fishmeal without affecting the growth performance of gilthead seabream. However, the highest final weight was observed on the CON diet. These results aren't consistent with previous studies that show better growth performance in organically reared fish than in conventionally reared fish [17]. However, the survival rates of the organic groups in this study were not significantly different from those of the conventional group, which differs from some previous studies that reported higher survival rates in organic fish [17]. It should be noted that the specific ingredients (remains of trout, visceral Iberian pig, and insects) used in the organic diets can significantly impact the growth and composition of fish, as demonstrated by the differences observed between the different organic and control diets in this study.

According to Di Marco's studies [17] on the organic farming of European seabass and gilthead seabream, researchers found that organic fish exhibited better growth performance, as indicated by their lower feed conversion ratio and higher metabolic status, supported by their protein and energy profiles and higher hepatosomatic index (HSI), but a lower mesenteric fat index and higher lipid content in organic European seabass fillets. These differences were attributed to the composition of the feeds provided to the different groups, as shown in a similar study by Trocino *et al*. [16] on European seabass and Mente *et al*. [14] on gilthead seabream. Only a few studies have examined the substitution of fishmeal in organic diets, and most of them have focused on freshwater species.

Lunger *et al*. [26,27] used organic yeast (NuPro) to replace up to 25% of FM in the cobia and tilapia feeds, respectively, and found that it did not affect the growth or feed conversion rate.

The FCR values obtained in this study indicate that fish could probably be fed slightly less while maintaining the same growth at the trial temperature. However, FCR is consistent with the temperature, growth, and feed intake shown. Aquaculture and animal byproductsprotein-based ingredients in organic aquaculture have yet to be well studied. More research is needed to determine their effects on product quality and other aspects, such as histology and digestibility, in organic aquaculture. Further research is required to optimize the formulation of organic feed and evaluate the long-term effects of organic farming on fish health and growth.

Experimental organic diets positively affected the digestibility of dry matter, calcium, gross protein, and gross energy in gilthead seabream (Table 7).

Some organic diets had higher apparent digestibility coefficients (ADC) for these nutrients than the CON diet, indicating that fish could better digest and utilize them. Previous studies have had different results on the effect of organic feeds on digestibility. For example, the study conducted by Amirkolaie *et al.* [28] revealed that substituting fishmeal with poultry by-product meal (PBM) reduced the digestibility of dry matter, fat, and protein in rainbow trout. Other studies, instead, reported that aquafeeds formulated with alternative protein sources had higher ADCs for protein and energy in various fish species, such as tilapia and catfish [8]. In general, these studies suggest that organic raw materials have the potential to improve the digestibility and utilization of nutrients in aquafeeds, which can lead to improved growth and health in farmed fish. However, digestibility is influenced by the raw ingredients and must be studied separately.

Studying the dynamics of protein hydrolysis in experimental diets for gilthead seabream is essential to aquaculture nutrition. The results of this study show that the rate of protein hydrolysis varied between the experimental diets, which indicates that different organic raw materials may have other effects on protein hydrolysis and subsequent nutrient availability. The current study provides new information on the impact of different organic raw materials on protein hydrolysis in gilthead seabream diets. The study found that the diet with the highest hydrolysis rate was IBE, which contained visceral Iberian pig meal as a protein source. This finding is consistent with previous studies showing variations in protein hydrolysis rates among raw materials [29].

As is known, EAAs cannot be synthesized and must be obtained through diet, making them essential for optimal growth and health. Retention efficiency can vary depending on the specific EAA and the composition of the diet.

The results did not indicate significant differences in the retention efficiency of EAA in the various diets except for methionine (Met). Met retention efficiency was significantly higher on the INS diet. Mente *et al.* [25] reported differences in the retention efficiency of essential amino acids in European seabass fed with different organic diets. These findings differ from previous studies that have reported differences in the retention efficiency of EAA in fish fed a blend of animal and plant protein diets. Monge-Ortiz *et al.* [30] reported that the retention ratio of seven EAA in the whole-body profile of fish varied significantly between the diets of *Seriola dumerili*. Overall, the findings from the present study suggest that the retention efficiency of EAA in fish-fed organic diets can vary depending on the type and composition of the organic raw materials used. Methionine is a limited amino acid in fish, and its higher retention in CON and INS diets (Table 8) could affect the PPV value of Table 5.

The gilthead seabream in the study did not show significant lipid accumulation or nucleus displacement caused by vacuoles, indicating a typical healthy morphology. The results indicated that the experimental organic diets significantly affected the histological measures of the liver in gilthead seabream. The diameter of the hepatocyte was significantly reduced in fish-fed organic diets compared to the CON diet. The reduced hepatocyte diameter in fish-fed organic diets may indicate decreased lipid accumulation and improved liver health. Furthermore, the diameter of the nuclei was significantly reduced in fish-fed organic diets compared to the control diet, which may indicate improved liver function. This finding is consistent with a study by Mourente *et al.* [31], which investigated the effects of plant-based diets on the liver morphology of seabass and found that the diameter of the hepatocyte was significantly reduced in fish fed plant-based diets. These findings suggest that organic diets can positively affect gilthead seabreams′ liver health and morphology. To date, no studies have investigated the histological effects of organic diets on the fish liver.

The study results showed that the different organic diets significantly affected the distal measurements of the gut in gilthead seabream. The diets affected the SL, ML, SML, VL, and VT. Specifically, gilthead seabream fed the CON and IBE diets had significantly longer SL and VL than those fed the TRO and INS diets. Regarding ML, SML, and VT, gilthead seabream fed the IBE diet had significantly longer measurements than those fed the TRO and INS diets. Finally, LP was considerably longer in gilthead seabream fed the CON and IBE diets than in those fed the TRO and INS diets. Limited studies have investigated the effects of different organic diets on intestinal measurements in gilthead seabream. However, Fronte *et al.* [32] examined the impact of different nitrogen-rich ingredients, such as hydrolyzed fish protein and autolyzed yeast, on the histological intestinal morphology of gilthead seabream (*Sparus aurata)* and found that villi branching and thickening were significantly affected by diets.

Furthermore, according to Torrecillas *et al.* [33], who investigated the effects of a vegetable-based diet on the gut health of seabass and found that villi height and epithelial thickness of the gut were significantly reduced in fish fed the vegetable-based diet compared to those fed a fishmeal-based diet. These findings suggest that different organic diets can dramatically affect the health and morphology of the intestinal tract of gilthead seabream.

Based on the results presented in the study, it can be reported that gilthead seabream fed the INS, TRO, and IBE diets had lower final weights than those fed the CON diet. However, survival rates were similar among all diets. Gilthead seabream fed the TRO, INS, and IBE diets had a lower protein content than those fed the CON diet. On the contrary, the fat content was higher in the TRO and IBE diets compared to the CON diet. The retention efficiencies of fat were also lower in the TRO and IBE diets than in the CON and INS diets. The apparent digestibility coefficients (ADC) of dry matter, gross protein, and gross energy were higher in the INS and IBE diets compared to the CON and TRO diets. The retention efficiency of EAA is mostly the same among diets except for Met. The IBE and TRO diets had lower retention efficiency for Met than the CON and INS diets. Gilthead seabream fed the TRO and INS diets had smaller nuclei and hepatocyte diameters in their livers than those fed the CON and IBE diets. Gilthead seabream fed the TRO and INS diets had shorter distal intestinal measurements than those fed the CON and IBE diets.

Conclusion

In summary, the results of this study suggest that the total replacement of fishmeal with organic raw materials, such as the remains of rainbow trout, the viscera of Iberian pigs, and insects, has some advantages in terms of digestibility, histology, and growth performance. However, the CON diet is still optimal regarding overall nutritional composition and amino acid retention. In practice, the results show that TRO, IBE, and INS meals can be used to replace fishmeal without harming growth performance, nutrient utilization, or intestinal health. Further research and optimization of organic diet formulations may be necessary to improve and maximize efficiency, but organic ingredients have a promising future in the aquaculture of seabream farming. These findings provide valuable information on the effects of different organic diets on digestibility and gilthead seabream liver and intestinal morphology. They could be used to improve sustainable and healthy aquaculture practices.

Acknowledgement

The first author was supported by a complete grant from the Ministry of Higher Education of the Arab Republic of Egypt and the research project had been developed with the collaboration of the Biodiversity Foundation (Spanish Ministry for Ecological Transition and the Demographic Challenge), through the Pleamar Program, co-financed by the European Maritime and Fisheries Fund (EMFF).

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Chapter 3.

Organic Ingredients as Alternative Protein Sources in the Diet of Juvenile Organic Seabass (*Dicentrarchus labrax*)

Organic Ingredients as Alternative Protein Sources in the Diet of Juvenile Organic Seabass (*Dicentrarchus labrax*)

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Animals, 2023

Tefal, E.; Jauralde, I.; Martínez-Llorens, S.; Tomás-Vidal, A.; Milián-Sorribes, M.C.; Moyano, F.J.; Peñaranda, D.S.; Jover-Cerdá, M. Organic Ingredients as Alternative Protein Sources in the Diet of Juvenile Organic Seabass (*Dicentrarchus labrax*). *Animals* **2023**, *13*, 3816. https://doi.org/10.3390/ani13243816.

Abstract

The use of organic ingredients as a source of protein in aquaculture diets has gained significant attention due to the growing demand for organic seafood products. This study aimed to evaluate the potential for the use of organic ingredients as protein sources in the diet of juvenile organic seabass (*Dicentrarchus labrax*). A total of 486 juvenile seabass with an average weight of 90 g were fed six diets containing varied organic proteins. The control group (CON) was fed a diet with conventional fishmeal from sustainable fisheries as the primary protein source. The other five groups were fed diets with different compositions: organic Iberian pig meal byproduct (IB diet), a combination of organic Iberian pig meal byproduct and insect meal (IB-IN diet), a mix of organic Iberian pig meal byproduct and organic rainbow trout meal byproduct (IB-TR diet), a blend of organic rainbow trout meal byproduct and insect meal (TR-IN), and a mixed diet containing all of these protein sources (MIX diet). Over a 125-day feeding trial, growth performance, feed utilisation, feed digestibility, and histological parameters were assessed. The results showed that the fish fed the control diet had the highest final weight and specific growth rate, followed by the fish fed the TR-IN and IB-TR diets. The IB-TR diet had the highest apparent digestibility coefficients (ADCs) for protein, while the TR-IN diet had the lowest. Histological analysis revealed that fish fed the control diet had the largest nucleus diameter and hepatocyte diameter. Use of IN seems to penalise performance in several ways. Fish fed diets containing insect meal grew less, and those diets had lower digestibility. Fish fed the TR and IB diets grew at rates near that of the control, and the feed had acceptable digestibility.

Keywords: organic aquaculture; alternative protein sources; organic ingredients; seabass; fish nutrition; aquaculture sustainability

Introduction

The term “organic production” refers to a farming and food-production approach that incorporates optimal environmental practices, promotes extensive biodiversity, conserves natural resources, upholds stringent standards for animal welfare, and aligns with the preferences of consumers seeking products created using environmentally friendly methods [1]. Organic aquaculture is a modest but growing part of the global food-supply chain [2]. Its production methods [3] were adopted due to the increasing interest in sustainable resource utilisation [4,5]. Organic aquaculture can be carried out using various technologies, including recirculating aquaculture systems (RAS), net pens, cages, raceways, and tanks [6]. The appropriate fish stocking density in RAS qualifies these systems for use in organic production, providing safe conditions for most fish in terms of animal welfare and biosecurity concerns [7]. Nevertheless, transitioning from conventional to organic aquaculture involves an intricate and multifaceted process, encompassing considerations related to consumer safety, ecological and environmental impacts, socioeconomic factors, and animal welfare [8,9]. The argument over the use of organic feeds for organic aquaculture is still ongoing because a balance must be struck between the realities of the supply of sources for aquafeeds and the fundamental principles of organic food production. Additionally, feeds must support animal health and growth, provide a final edible product of excellent quality, have a low impact on the environment, and be balanced to meet the nutritional needs of the farmed species [10].

In 2020, the EU27’s total organic aquaculture production was approximated at 74,032 tonnes, constituting 6.4% of the EU’s total output. This production marks a 60% increase from 2015 (46,341 tonnes at the EU 27 level in 2015), primarily due to increased production of organic mussels [11]**.** One of the main species produced organically in the Mediterranean is European seabass, the production of which increased from 2000 tonnes in 2015 to 2750 tonnes in 2020. Greece is the leading EU producer of this fish [11]. Economic considerations such as increased production expenses and increased retail costs have dissuaded both farmers and consumers, limiting the growth of organic production of seabass [12]. Research on consumer preferences shows that organic seabass production in the Mediterranean is economically promising [13,14], but appropriate marketing strategies still need to be developed [15,16]. Furthermore, a major obstacle to expanding organic aquaculture production is the lack of organic feeds, particularly for carnivorous species. The limitations imposed by the EU organic regulations make it difficult to find organic feed ingredients that are rich in protein and thus to design well-balanced organic diets [17–20].

More research is required on the organic cultivation of seabass. Previous studies reported that organic fish show improved growth performance, lower feed-conversion ratios, and increased metabolic rates compared with fish grown in conventional aquaculture [18]. However, other studies have found no differences in stress and immunological indices between fish grown in organic and conventional aquaculture [18,21].

The general principles of ecological production, such as the development of processes that are based on environmental systems and that use the system’s natural resources, the restricted use of synthetic substances, and the limited use or non-use of genetically modified organisms (GMOs), apply to organic aquaculture, with some additional limitations regarding the availability of organic resources [22]. The relevant regulatory limitations stipulate a maximum of 60% organic plant ingredients and the absence of synthetic amino acids [23]. Continual efforts are required to identify alternative sources of nutritional protein and lipids for organic feeds in organic aquaculture, with a focus on minimising the utilisation of fishmeal (FM) and fish oil in such feeds. However, there is a need to focus on the quality and certification of alternative ingredients for use in organic aquaculture. Research is still being done to investigate novel alternative formulations of ingredients and the quality of the resulting products [1].

Many researchers have studied the effects of substituting FM with plant-based proteins [24–29]. Completely replacing animal proteins with plant proteins has generally not been successful due to concerns about antinutrients, changes in amino-acid absorption, potential micronutrient deficiencies, and immune suppression [30–32]. Other potential feed sources, excluding plant proteins, include microbiological organisms (bacteria, microalgae, fungi), byproducts from terrestrial animals (processed animal protein (PAP), blood meal), annelid worms obtained from wild harvesting and cultivation, and the larvae and pupae of insects [33–35].The utilisation of animal byproducts is made possible by PAP, a key ingredient in feeds [36]. According to several studies, insects can be used as a source of protein for fish [37–40]. European seabass can be fed insect meal from *Tenebrio molitor* at varying concentrations without adversely affecting growth performance, according to a feeding assay [41]. Byproduct meals can be highly appealing due to their competitive pricing compared to fish meal, making them a potentially interesting and cost-effective option [42].

Due to regulatory restrictions, it is difficult to find enough organic protein sources suitable for seabass, one of the main carnivorous fish produced in Europe. Transformed animal proteins (TAPs) from non-ruminant animals, whose use is permitted in conventional aquaculture (RD 578/2014), as well as insects (Regulation EU 893/2017), are suggested. The use of organic-derived TAPs in organic aquaculture does not violate any regulations and facilitates the formulation of organic aquaculture feed without the need for captured FM, relying solely on the recovery of byproducts from organic aquaculture.

On the other hand, there has been considerable interest in the use of in vitro assays to evaluate the digestibility of a prospective feed product for aquatic species, such as fish, prawns, and molluscs [43]. The in vitro digestibility test is appropriate for preliminary research. It allows many samples to be analysed because it is inexpensive, has no ethical restrictions, and is reasonably simple to carry out [44]. It also allows the conduction of controlled experiments to investigate how proteins, lipids, and carbohydrates in feed items are hydrolysed [43]. Research on fish digestion in vitro is still in its infancy, based on the number of relevant publications.

The present work aimed to provide a 100% organic diet for seabass, one of Europe’s most important marine aquaculture species, using alternative organic raw materials such as insects, byproducts from Iberian pigs, and rainbow trout remains. This research may support a dramatic improvement in organic aquaculture.

Materials and methods

Rearing system

Ethics approval

In accordance with Royal Decree 53/2013 and European Directive 2010/63/EU concerning the protection of animals used for scientific research, the experimental protocol underwent review and received approval from the Ethics and Animal Welfare Committee of the Universitat Politècnica de València (Official Bulletin No. 80 of 06/2014) to ensure the welfare and minimise the suffering of animals. Ethical approval was granted on 27 January 2022.

System for Rearing

The trial was conducted in 18 cylindrical fiberglass tanks, each with a volume of 1750 L, as part of a saltwater recirculating system with a total capacity of 75 m^3. The system was equipped with a rotating mechanical filter, a gravity biofilter with a capacity of 6 m^3, and a skim (September to January). A heat pump was used to ensure that the water temperature remained constant (20.9 °C), and all tanks had aeration. The dissolved oxygen level was 7.7 mg L^{-1}, and salinity was 31.3 g L^{-1}. The pH was maintained at 8.0, with nitrates (NO^{-3}) at a concentration of 33.2 mg L^{-1}, nitrites (NO^{-2}) at 0.13 mg L^{-1}, and ammonium (NH^{+4}) at 0.03 mg L^{-1}. The photoperiod was natural (11 h), and the lighting was the same in all tanks.

Fish

Juvenile organic seabass from the fish farm Sonrionansa S.L. situated in Pesues (Cantabria, Spain) were delivered to the Universitat Politècnica de València and distributed among experimental tanks. Before the feeding experiment, a 15-day acclimatisation period was provided to allow all fish to adapt to the laboratory conditions. There were 486 fish, with an average weight of 90 g, distributed throughout the 18 test tanks (27 fish per tank). The experiment was carried out over 125 days.

Diets and feeding

The proximal composition of the raw materials is shown in Table 1. Six diets were tested in triplicate: (1) a control diet containing FM provided for sustainable fisheries as a protein source (CON); (2) a diet in which the protein source was composed of Iberian pig meal byproduct (diet IB); (3) a diet containing organic Iberian pig meal byproduct and organic insect meal (diet IB-IN); (4) a diet based on organic Iberian pig and organic rainbow-trout byproduct meal (diet IB-TR); (5) a diet with organic rainbow trout byproducts and organic insect meal as protein sources (diet TR-IN); and (6) a MIX diet containing organic insect meal, organic rainbow trout meal and organic Iberian pig byproduct meal (Table 2). The nutritional compositions of all the diets are represented as the means of five separate analyses conducted during each feed-manufacturing cycle. Formulations were initially designed with different amounts of raw ingredients to maintain a consistent composition of 45% crude protein (CP) and 20% crude lipid (CL); challenges in effectively mixing certain ingredients led to variations in some of these values (Table 2).

All diets were manufactured at the Universitat Politècnica de València using a semi-industrial twin-screw extruder (CLEXTRAL BC-45, Firminy, St Etienne, France), following the following processing parameters: screw speed of 100 rpm, pressure range of 40–50 atm, and temperature of 110 °C. Calcium phosphate and organic vegetable amino acids were included in diets as supplements (lysine and methionine). Diet formulation and manufacture were carried out with organic raw materials labelled and approved by Regulation (EU) 2018/848.

Table 1. The characteristics of the raw materials. Macronutrient composition of the different ingredients used in the study (% m.s.).

Raw Materials (%)	Fishmeal	Insect Meal	Remains of Rainbow Trout	Iberian Pork Viscera	Organic Wheat	Organic Soybean Meal
Dry matter	91.9	92.6	95.71	92.6	92.3	92.3
Crude protein	73.9	37.6	76.71	53.0	12.7	43.1
Crude lipid	11.2	28.5	17.36	28.6	1.3	9.3
Ash	14.3	13.9	11.38	3.8	1.7	6.3
Gross energy (kJ/g) **	22	24	24	27	18	21

** Gross energy (kJ/g) = [51.8 × (%C/100)) − (19.4 × (%N/100)].

Table 2. Ingredients and proximal composition of the diets utilised in the growth experiment.

Ingrdients (g kg^{-1})	CON	IB	IB-IN	IB-TR	TR-IN	MIX

Raw materials (g kg^{-1})						
Fishmeal	304					
Insect meal			214		215	143
Remains of rainbow trout				261	261	143
Iberian pork viscera		351	214	138		143
Organic wheat	213	147	75	152	80	100
Organic soybean meal	325	325	325	325	325	325
Fish oil [a]	50	62	57	49	44	51
Organic soybean oil [b]	83					
Calcium phosphate	10	25	25	25	25	25
Vegetable methionine [c]	5	40	40	20	20	30
Vegetable lysine [d]		40	40	20	20	30
Vitamins [e]	10	10	10	10	10	10
Nutritional composition (% DM)*						
Dry matter	87.9	88.9	84.4	89.1	92.7	92.7
Crude Protein	44.3	42.8	44.1	43. 7	45.1	45.6
Crude lipid	19.8	21. 6	19.7	21.0	20.0	19.8
Ash	8.3	6.8	8.9	9.4	11.1	9.4
Gross energy **	22.4	23.4	23.5	23.4	23.1	23.2
Digestible energy	77.7	83.9	76.5	83.4	73.3	78.3

[a] Fish oil (Industrias Afines, SRL (Arpo), Polgono industrial A Veigadaa, Ra as Baloutas, de Abaixo, 24, 36416, Pontevedra, Spain). [b] Organic soybean oil (Clearspring Ltd., Acton Park Estate, London W3 7QE, UK). [c] Vegetable methionine (Adibio S.L.|Edificio Galileo, C/Enebros 74, 2ª planta|44002 Teruel (Spain). [d] Vegetable lysine (Adibio S.L.|Edificio Galileo, C/Enebros 74, 2ª planta|44002 Teruel (Spain). [e] Vitamin-and-mineral mix (g kg^{-1}): Premix: 25; Choline, 10; DL-a-tocopherol, 5; ascorbic acid, 5; $(PO_4)_2Ca_3$, 5. Premix composition: retinol acetate, 1,000,000 IU kg^{-1}; calciferol, 500 IU kg^{-1}; DL-a-tocopherol, 10;m menadione sodium bisulfite m menadione, 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. CON: control; IB-IN: Insect-Iberic; IB: Iberic; TR-IN: Trout-Insect; IB-TR: Trout-Iberic; and MIX: Insect-Iberic-Trout. * The nutritional composition values represent the means of five analyses conducted during each cycle of feed production throughout the experiment. ** Gross energy (kJ/g) = $[51.8 \times (\%C/100)) - (19.4 \times (\%N/100)]$.

Each experimental diet was evaluated in three randomly distributed tanks. The fish were manually fed twice a day at 9:00 and 17:00, six days per week (from Monday to Saturday).

The fish were fed until they reached satiety, and the pellets were administered gradually. Daily monitoring and weighing occurred every 30 days before anaesthetising the fish were anaesthetised with clove oil, which contained 87% eugenol (Guinama®, Valencia, Spain), at a concentration of 10 mg L^{-1} of water.

The aim of this process was to evaluate the growth of the fish throughout the experiment, define growth parameters, and assess the overall health of the fish. The fish were starved the day before they were weighed. Ten fish were collected at the start of the experiment and preserved at −30 °C for subsequent analysis of their body composition. Three specimens from each tank were randomly selected for sampling and pooling to determine the approximate composition and amino acid content of their bodies.

Analysis of Nutritional Composition and Amino Acids

The diets and their approximate composition (Table 2), as well as the whole fish, were examined using the methods described in [45] and analysed for the following metrics: dry matter (105 °C to constant weight); ash (incinerated at 550 °C for five hours); crude protein (determined by the direct combustion method DUMAS using LECO CN628, Geleen, The Netherlands); and crude lipid (extracted with methyl-ether using ANKOMXT10 Extractor (Macedon, NY, USA)). Each analysis was carried out in triplicate.

A Waters HPLC system (Waters 474, Waters, Milford, MA, USA) composed of two pumps (Model 515, Waters), an autosampler (Model 717, Waters), a fluorescence detector (Model 474, Waters), and a temperature-control module was used to analyse the levels of amino acids (AA) in the diets and in the fish using the procedure previously described by Bosch *et al*. 2006 [46].

Before hydrolyzation, aminobutyric acid was introduced as a internal standard. AQC was used to derivatise AA (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). After oxidation with performic acid, methionine and cysteine were identified individually as methionine sulphone and cystic acid. AA was converted to methionine and cystine after it was separated with a reverse-phase C-18 column by Waters Acc—Tag (150 mm 3.9 mm). Table 3 shows the essential amino acids (EAA) content of the experimental diets. All amino acid analyses were carried out in duplicate.

Table 3. Composition of essential and non-essential amino acids in experimental diets.

Diets	**CON**	**IB**	**IB-IN**	**IB-TR**	**TR-IN**	**MIX**

Essential amino acids (g 100 g^{-1})						
Arginine	3.01	2.27	2.25	2.07	2.42	2.67
Histidine	1.13	2.13	1.14	0.99	1.20	1.10
Isoleucine	1.71	2.04	1.83	1.69	1.96	1.85
Leucine	3.22	3.72	3.18	3.02	3.10	3.24
Lysine	2.83	2.72	2.50	2.59	2.70	2.65
Methionine	0.78	0.51	0.74	0.97	0.90	0.56
Phenylalanine	1.90	2.17	1.94	1.73	1.91	1.93
Threonine	1.65	1.76	1.47	1.35	1.29	1.64
Valine	2.29	2.71	2.44	2.15	2.30	2.44
Non-essential amino acids (g 100 g^{-1})						
Alanine	2.31	2.64	2.33	2.08	2.25	2.54
Aspartic acid	3.89	4.14	4.28	3.69	4.14	3.96
Cysteine	0.47	0.33	0.42	0.48	0.47	0.32
Glutamic acid	6.72	6.94	5.78	6.13	5.85	6.19
Glycine	2.35	3.08	2.16	2.53	2.54	2.50
Proline	2.05	2.33	2.14	2.02	2.38	2.24
Serine	1.78	3.32	2.24	1.74	2.13	1.87
Tyrosine	1.43	1.57	1.87	1.21	1.85	1.70

CON: control; IB-IN: Insect-Iberic; IB: Iberic; TR-IN: Trout-Insect; IB-TR: Trout-Iberic; and MIX: Insect-Iberic-Trout.

Indices of Growth

At the end of the trial, the nutrient efficiency indices and growth were determined. Metrics included the survival rate (SR), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), and protein efficiency ratio (PER), considering each tank as an experimental unit. All fish were weighed. Additionally, the productive protein value (PPV%) and productive fat value (PFV%) were calculated. These parameters were calculated using the following equations:

$$\text{SGR} = 100 \times \ln(\text{final weight}/\text{initial weight})/\text{days}$$

$$\text{FI (g 100 g fish} - 1 \text{ day} - 1) = 100 \times \text{feed consumption (g)}/\text{average biomass (g)} \times \text{days}$$

$$\text{FCR} = \text{feed consumption (g)}/\text{biomass gain (g)}$$

$$\text{PER} = \text{biomass gain (g)}/\text{protein intake (g)}$$

PPV% = Protein retained (final fish protein × Final biomass (g)) × 100 − Initial fish protein × initial biomass (g)/Protein ingested (kg of ingested feed × % crude protein).

PFV% = Fat retained (final fish fat × Final biomass (g)) × 100 − Initial fish fat × initial biomass (g)/fat ingested (kg of feed × % crude fat)

Digestibility Assay

The digestibility test was performed after the growth experiment ended and was carried out in triplicate, in three tanks. Fifteen seabass were randomly placed in each experimental tank (190 L fibreglass tank, 88 cm high, 62 cm wide, and 188 cm deep) in a semi-closed recirculating system based on the Guelph system (the faecal material being collected in a settling column). The water flow velocity was altered to reduce the settling of faeces in the drainpipe and increase the recovery of faeces in the settling column.

The fish received one meal per day at 10:00 AM. The diet was offered so as to reduce waste while the fish were actively feeding. The drainpipe and the settling column were dusted an hour after feeding to prevent faeces from being contaminated by column diets. The faeces were gravity-collected in a plastic container from the base of the settling column 6–7 h after feeding.

After collection, the faeces were weighed and dried in a 60 °C oven for 48 h before analysis. Subsequently, they were preserved in sealed plastic containers and analysed for nutritional components and inert markers. Chromic oxide (Cr_2O_3) was used (5 g kg^{-1}) as an inert and indigestible marker. An atomic absorption spectrometer was used to determine the amount of chromium oxide in diets and faeces after acid digestion (Perkin Elmer 3300, Perkin Elmer, Boston, MA, USA). Additionally, analyses were conducted for crude protein, dry matter, calcium, energy, and phosphorus in diets and faces. All analyses were performed in triplicate.

The apparent digestibility coefficients of the diet (ADC) were determined using Cho method [47]. The ADCs of the dry matter (ADCdm, %) of the diets were determined per Equation:

ADCdm % = 1 − (% Cr_2O_3 in diet/% Cr_2O_3 in faeces)

The percentage of ADCs for each dietary nutrient (protein, energy, calcium, and phosphorus) was calculated using Equation:

ADCnut = 1 − ((marker diet/marker faeces × (nutrient faeces/nutrient diet)).

The variables “nutrient diet (g kg^{-1})” and “nutrient faeces (g kg^{-1})” in this equation indicate the amounts of a nutrient (such as protein or energy) in the diet and the faeces, respectively. The measurements “marker diet” (g kg^{-1}) and “marker faeces” (g kg^{-1}) indicate the amount of marker in the diet and the faeces, respectively.

In vitro hydrolysis assay

The in vitro hydrolysis trial was conducted under conditions that simulated the digestive system of juvenile European seabass [48]. Ten juvenile seabasses with an average weight of 100 g were used and sampled six hours after feeding to ensure the presence of enzymes in both the stomach and the intestine. The fish were euthanised in ice-cold water with a small quantity of clove oil, which acted as an anaesthetic. Subsequently, the fish were immediately dissected to extract the digestive tract. The digestive tract was divided into two parts: (1) the proximal intestine, which encompassed the diffuse pancreas and pyloric caecum, and (2) the stomach. These tissues were utilised to create extracts for measuring protease activity. The methods used were as follows: acid protease was measured by tyrosine release from haemoglobin hydrolysis at pH 2.5 [49]; alkaline protease was measured by tyrosine release from casein at pH 8.5 [50]; and amylase was measured during the preliminary evaluation of the enzymes of the juvenile European seabass at pH 7.5 [51]. The extracts were prepared by mechanical homogenisation of the tissues in distilled water (1:10 *w*/*v*) and centrifugation (3220× *g*, 20 min, 4 °C). The supernatant was then filtered through a dialysis system with a MWCO of 10 kDa (Pellicon XL, Millipore, Burlington, Massachusetts, USA), and the concentrated extracts were freeze-dried until they were required for the assays. The activities of acid protease in the stomach (pepsin) and total intestinal alkaline proteases present in the extracts were measured using the methods described in refs. [48,49]. Protease activity levels were used as indicators to estimate the amount of extracts required to provide physiological enzyme-substrate ratios in the assays. These ratios were calculated considering, on one hand, the average total production of enzyme measured in several fish in relation to their live weight, and on the other, the average intake per meal of fish of such a size, a value obtained from commercial ration tables.

Based on these findings, the average enzyme production was estimated as follows: acid protease, 37.7 U g^{-1} weight; and alkaline protease, 24.7 U g^{-1} weight. The conditions are given in Table 4.

Table 4. The specific conditions of the protein hydrolysis assay.

	Acid stage	Alkaline stage
E:S ratio (U/mg protein)*	4.0	8.5

pH	3.5	8.5
Time (hours)	1.5	3.5
Temperature (ºC)	25	25

* E:S ratio: enzyme/substrate ratio

Three assay replicates were conducted for each diet, and a blank sample was also included. The blank sample involved deactivating the enzymatic extracts through heat treatment before adding them to the bioreactors. This step enabled measuring the amino acid content in the extracts and the diet.

Histological analysis of the liver

The liver was collected from three fish per tank after the growth experiment ended. Samples were preserved in phosphate-buffered formalin (4%, pH 7.4). According to typical histological procedures, all the formalin-fixed tissues underwent regular dehydration in ethanol, were conditioned in ultra-clean environments, and were embedded in paraffin. Transverse sections from each paraffin block were taken using a Shandon Hypercut microtome, then stained for haematoxylin and eosin analysis.

One hundred sections of the liver were examined using an Eclipse E400 Nikon light microscope from Izasa S.A. in Barcelona, Spain. To determine the effects of different feeds on the liver, the diameters of hepatocytes and nuclei were measured [52,53].

Statistical Analysis

All data were checked for normal distribution and homogeneity of variances. Using the Statgraphics® Plus 5.1 statistical programme (Statistical Graphics Corp, Rockville, MO, USA), various growth and nutrient indices, retention of AA, ADC, in vitro hydrolysis, and histological measurements were analysed using analysis of variance with a Newman-Keul test for multiple comparisons. The initial covariate weight was used to analyse growth indices. The findings are represented as means with standard error (SEM, standard error of the mean). The significance level was established at $p < 0.05$.

Results

3.1 Growth and Nutritional Parameters

The evolution of fish weight throughout the experiment showed a general increase regardless of the experimental group. Figure 1 illustrates that the control group exhibited the highest final weight, followed by the diets containing organic fishmeal (TR-IN and IB-IB-TR), while the remaining treatments displayed lower.

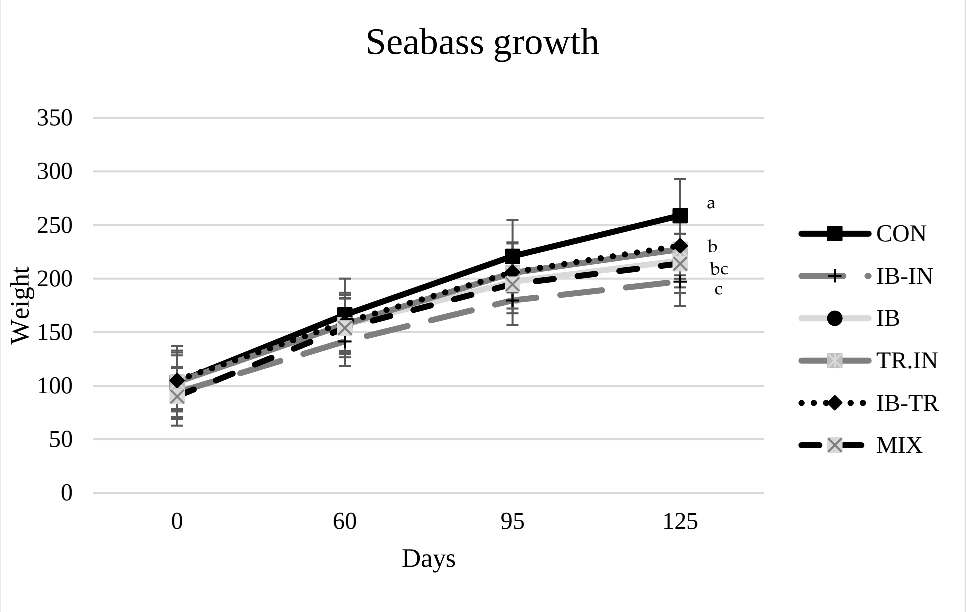

Figure 1. Evolution of the average weight of the fish during the experiment. Values represented as mean ± standard deviation (n=3). Different superscript in each sampling means significant differences ($p < 0.05$). Test Newman-Keuls. CON: control; IB-IN: Insect-Iberic; IB: Iberic; TR-IN: Trout-Insect; IB-TR: Trout-Iberic; and MIX: Insect-Iberic-Trout.

The final weight of the fish (FW) and the SGR were affected by the composition of the diet (Table 5). Fish fed the control diet had the highest final weight and SGR (258.7 g, 1.16%/day, respectively), followed by fish fed diets TR-IN and IB-TR, which obtained grew more than fish fed the IB-IN diet. Survival, FI, and FCR did not show significant differences at the end of the experiment.

Table 5. Growth and nutritional parameters of European seabass fed experimental diets.

Diets	CON	IB	IB-IN	IB-TR	TR-IN	MIX	SEM
Initial weight (g)	92.0	91.2	88.2	89.5	91.2	90	2.15
Final weight (g)	258.7^{a}	216.7^{bc}	197.3^{c}	230.7^{b}	227.3^{b}	214.0^{bc}	6.55
Survival (%)	95.0	97.3	94.0	95	96.3	97.7	2.82
SGR (%day^{-1})^{1}	1.16^{a}	1.06^{bc}	1.01^{c}	1.10^{b}	1.09^{b}	1.05^{bc}	0.018
FI (g100 g^{-1} fish day^{-1})^{2}	0.95	0.95	1.02	0.96	0.99	1.01	0.039

FCR[3]	2.02	2.42	2.64	2.36	2.38	2.12	0.174
PER[4]	0.91	0.82	0.82	0.82	0.75	0.80	0.088

Values represented as mean ± standard error (n=3). Different superscripts in the same raw means significant differences ($p < 0.05$). Test Newman-Keuls. CON: control; IB-IN: Insect-Iberic; IB: Iberic; TR-IN: Trout-Insect; IB-TR: Trout-Iberic; and MIX: Insect-Iberic-Trout.

[1] Specific growth rate (SGR) = 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 consumption (g) /biomass gain (g).

[4] Protein efficiency ratio (PER) = biomass gain (g) / protein intake (g).

3.2 Body Composition and Nutrient Retention Efficiency

The nutritional composition of the whole body and the retention efficiency of protein and fat are shown in Table 6. No significant differences were observed for total dry matter, protein, fat, ash, protein efficiency retention, or fat efficiency retention (PPV and PFV). Table 6 also shows the efficiency of EAA retention. Except for arginine (Arg) and histidine (His), there were no statistical differences in the retention efficiency of EAA between the experimental diets. The highest retention for Arg was shown in fish fed the TR-IN diet, and the highest retention for His was observed in fish fed the TR-IN and IB-TR diets. Generally, the lowest EAA retention efficiency was found in fish fed the IB and MIX diets.

Table 6. Body composition and retention efficiencies of seabass at the beginning and after feeding with experimental diets.

	Initial	**CON**	**IB**	**IB-IN**	**IB-TR**	**TR-IN**	**MIX**	**SEM**
Dry matter	29.9	39.9	38.1	39.2	39.0	40. 6	38.8	1.3
Crude protein	18.0	16.8	16.5	16.7	16.9	17.4	16.3	0.4
Crude fat	8.4	19.2	18.0	18.6	18.0	19.4	18.4	1.1
Ash	3.1	3.1	3.4	4.3	3.5	3.1	3. 5	0.4
PPV [1]		15.9	13.7	12.8	16.9	15.9	13.7	1.2
PFV [2]		64.9	56.0	59.4	58.0	64.3	61.4	4.4
Retention efficiencies essential amino acids								
Arginine		20.3^{ab}	14.7^{b}	20.7^{ab}	26.95^{ab}	31.9^{a}	14.8^{b}	3.12
Histidine		14.5^{ab}	5.5^{b}	13.7^{ab}	16.1^{a}	21.2^{a}	13.0^{ab}	2.10
Isoleucine		17.6	11.3	11.7	18.2	17.7	12.9	1.92
Leucine		14.1	8.2	11.3	15.8	18.6	11.0	2.25
Lysine		19.9	12.9	18.7	23.7	27.2	15.7	3.29
Methionine		11.9	24.5	14.1	12.9	15.5	23.8	3.02
Phenylalanine		12.5	8.1	10.7	15.3	16.9	10.6	1.96
Threonine		17.5	12.0	15.6	20.9	28.2	14.4	2.23
Valine		14.7	9.0	11.7	17.4	18.9	11.6	2.63

Values represented as means (n = 3). SEM: standard error of the mean. Different superscripts in the same row indicate significant differences ($p < 0.05$) by the Newman-Keuls test. CON: control; IB-IN: Insect-Iberic; IB: Iberic; TR-IN: Trout-Insect; IB-TR: Trout-Iberic; and MIX: Insect-Iberic-Trout. [1] PPV%) = Protein retained (final fish protein × Final biomass (g)) × 100 − Initial fish protein × initial biomass (g)/Protein ingested (kg of ingested feed ×% crude protein). [2] PFV%) = Fat retained (final fish fat × Final biomass (g)) × 100 − Initial fish fat × initial biomass (g)/fat ingested (kg of feed × % crude fat). * Retention efficiencies of amino acids (%) = (fish amino acid gain (g) × 100)/amino acid intake (g).

3.3 Digestibility

The ADC for dry matter, phosphorus, protein, and energy of the experimental diets are shown in Table 7. ADCs for dry matter and phosphorus fish fed the IB diet showed the highest values (62.2 and 68.3%, respectively), and the TR-IN diet was the lowest (43.3 and 25.0%, respectively). The IB-TR diet was associated with the highest values (91.8%) of protein ADCs, and the TR-IN diet gave the lowest values (85.3%). A fish fed the IB diet showed the highest-energy ADCs.

Table 7. Apparent digestibility coefficients of dry matter and nutrients of seabass fed experimental diets.

	Diets						
ADC (%) *	**CON**	**IB**	**IB-IN**	**IB-TR**	**TR-IN**	**MIX**	**SEM**
Dry matter	55.6 ^{bc}	62.2 ^{a}	53.0 ^{c}	59.2 ^{ab}	43.3 ^{d}	53.5 ^{c}	1.8
Phosphorus	52.0 ^{bc}	68.3 ^{a}	44.5 ^{c}	57.8 ^{b}	25.0 ^{d}	57.0 ^{b}	3.0
Crude protein	88.3 ^{abc}	91.6 ^{ab}	87.1 ^{bc}	91.8 ^{a}	85.3 ^{c}	89.7 ^{abc}	1.4
Gross energy	77.7 ^{bc}	83.9 ^{a}	76.5 ^{c}	83.4 ^{ab}	73.3 ^{c}	78.3 ^{abc}	2.0

Values represented as means ($n = 3$). SEM: standard error of the mean. Different superscripts in the same row indicate significant differences ($p < 0.05$) by the Newman-Keuls test. CON: control; IB-IN: Insect-Iberic; IB: Iberic; TR-IN: Trout-Insect; IB-TR: Trout-Iberic; and MIX: Insect-Iberic-Trout. * ADC. ADCdm = 100 − [100 × (% Cr_2O_3 in diet/% Cr_2O_3 in faeces)]. ADCnut = 100 − [100 × (% feed marker/% faeces marker) × (% nutrient. energy. amino acid. or fatty acid in urine /% of nutrient. Energy. amino acid. or fatty acid in feed)].

3.4 In vitro hydrolysis assay

Results of amino acids released through the membrane after the dietary protein hydrolyzation sampling point are shown in **Figure 2**. The hydrolysis values were very similar among diets.

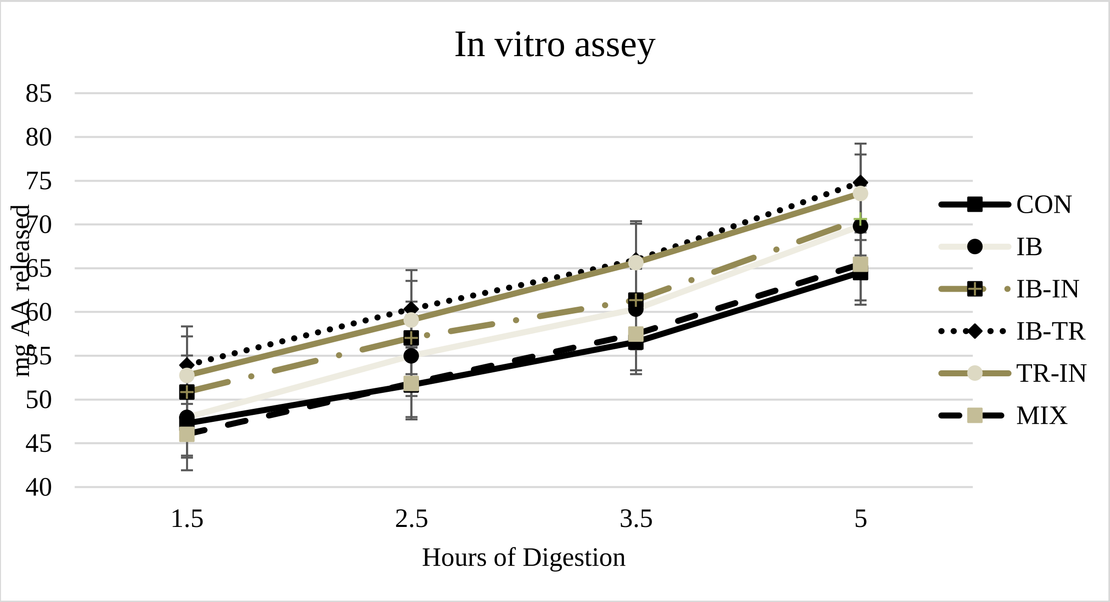

Figure 2. Release results of amino acids that cross the membrane after hydrolyzing from the protein of all experimental diets. Values represented as mean ± standard error (n=3). ($p < 0.05$). Test Newman-Keuls. CON: control; IB-IN: Insect-Iberic; IB: Iberic; TR-IN: Trout-Insect; IB-TR: Trout-Iberic; and MIX: Insect-Iberic-Trout.

The results were used to adjust the protein-hydrolysis dynamics in each diet. Using the following equations, various hydrolysis rates were estimated for a specific amount of protein. For example, for 100 mg of protein in the diet, the total hydrolysis time ranges from 9 to 12 h, as presented in Table 8. Furthermore, the steeper slope of the IB diet's adjustment line suggests that it hydrolyses faster. Table 8 shows the linear equations representing the relationships between the degree of protein hydrolysis (y) and the time (x) for each diet, along with the corresponding time points.

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Table 8. Linear adjustment equations for the protein-hydrolysis dynamics of the six experimental diets.

Diet	**Linear adjustment**	**Time (h)**
CON	$y = 4.9449x + 39.573$	12.22
IB	$y = 6.1678x + 39.016$	9.89
IB-IN	$y = 5.5398x + 42.66$	10.35
MIX	$y = 5,539x + 37.906$	11.21
IB-TR	$y = 5.9287x + 45.235$	9.24
TR-IN	$y = 5.9574x + 44.145$	9.37

Y: degree of protein hydrolysis, X: time. CON: control; IB-IN: Insect-Iberic; IB: Iberic; TR-IN: Trout-Insect; IB-TR: Trout-Iberic; and MIX: Insect-Iberic-Trout.

Based on the hydrolysis rate, it was noted that the IB-TR, TR-IN, and IB diets required less time for protein hydrolysis than the rest of the diets, especially when compared to the CON diet, which may have important implications when digestion transit rates and feeding frequencies are considered.

3.5 Histology of the liver

The liver histology results for seabass fed experimental diets is shown in Table 9 and shows differences in hepatocyte measurement (nucleus and diameter of the hepatocyte). Fish fed the CON diet showed the largest nucleus diameter, followed by fish fed IB-TR and TR-IN, then fish fed IB-IN, IB, and MIX diets, with smaller nucleus diameters. Likewise, fish fed the CON diet exhibited larger hepatocyte diameters than fish fed the experimental diets. No differences were found among the fish fed experimental diets.

Table 9. Histological assessments of the livers of seabass fed experimental diets.

Diets	CON	IB	IB-IN	IB-TR	TR-IN	MIX	SEM

Nucleus diameter (µm)	4.8^a	3.3^c	3.4^c	4.1^b	4.1^b	3.6^c	0.12
Hepatocyte diameter (µm)	11.2^a	7.7^b	8.2^b	8.9^b	8.4^b	8.6^b	0.24

Values represented as means (n = 100). SEM: standard error of the mean. Different superscripts in the same row indicate significant differences ($p < 0.05$) by the Newman-Keuls test. CON: control; IB-IN: Insect-Iberic; IB: Iberic; TR-IN: Trout-Insect; IB-TR: Trout-Iberic; and MIX: Insect-Iberic-Trout.

The liver histology of seabass fed experimental diets is shown in Figure 3. The histology of the liver for each treatment revealed hepatocytes with irregular morphology, absence of necrosis, and large nuclei displaced from the centre to peripheral areas. Lipid accumulations forming vacuoles in the hepatocyte cytoplasm were observed mainly in fish fed the CON, IB, and IB-IN diets.

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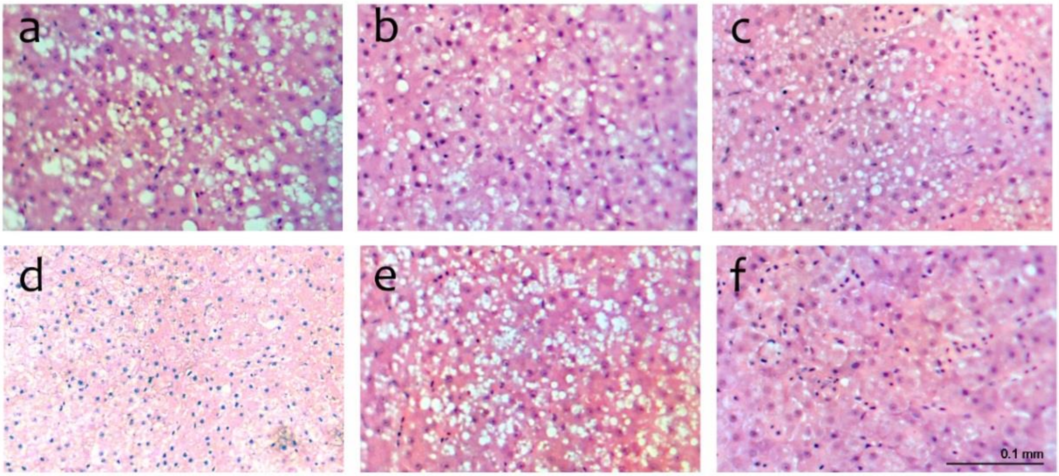

Figure 3. Histological details of the livers of seabass fed experimental diets (10×). (a) CON: control; (b) IB: Iberic; (c) IB-IN: Iberic- Insect; (d) IB-TR: Iberic-Trout (e) TR-IN: Trout-Insect; and (f) MIX: Insect-Iberic-Trout. Haematoxylin-Eosin staining.

Discussion

Formulating organic diets for carnivorous fish is a challenging task that requires careful consideration of fish nutritional requirements, organic regulations, and the availability and quality of organic feed ingredients. Perfectly balancing the amino acid profile is not always possible because organic feeding standards prohibit the use of synthetic amino acids. The organic amino acids available are precursors of amino acids and amino acid mixtures, making it impossible to achieve the perfect balance, as can be done with synthetic amino acids. The performance of fish fed organic alternative ingredients must be studied to optimise organic feeding practices. Improving the sustainability of carnivorous-fish production is one of the primary objectives of replacing fishmeal (FM), along with FM's high cost, which makes more affordable alternatives attractive.

Fish Performance

The results of this research suggest that organic ingredients, such as insects, byproducts of Iberian pigs, and rainbow trout remains, have potential as part of an initial step in completely replacing fishmeal in European seabass diets in aquaculture. The utilisation of byproducts results in highly competitive prices compared to fish meal, which may incentivise their use even when they produce suboptimal growth [42].

The findings of previous studies comparing conventional and organic feed in European seabass provide valuable insights. However, caution should be exercised when extrapolating those results for comparison to the present experiment due to differences in feed composition. For example, the study by Di Marco *et al*. (2017) [18] compared feeds containing different concentrations of fishmeal; the organic feed contained 56% fishmeal, and the conventional feed contained only 20%. Similarly, in experiments conducted with seabream, Mente *et al*. (2012) [5] observed improved growth in fish fed organic feed compared to those fed conventional feed. Again, however, the organic feed contained more fishmeal (63%) than the conventional feed (50%). These findings further support the notion that organic feed formulations enriched with fishmeal can positively impact growth performance in marine species. However, the higher growth seen in organic-fed fish in those experiments cannot be attributed solely to the origin, organic or conventional, of the fishmeal because the concentration is a significant factor. Fishmeal is known to be a protein source of high quality that includes essential amino acids and other nutrients necessary for optimal growth in marine species. Therefore, the higher percentage of fishmeal in the organic feed likely contributed to the observed growth improvement.

The outcomes of the present experiment indicate that the IB-IN, IB, and MIX diets resulted in lower final fish weights, suggesting a negative impact on the growth performance of European seabass. These results align with observations from a previous trial conducted by Tefal *et al*. (2023) [42], which investigated the total substitution of fishmeal (FM) with a new organic raw material in gilthead seabream feeding. Tefal *et al*. (2023) [42] found that organic diets can replace fishmeal in gilthead seabream feed without negatively impacting growth performance. Nevertheless, the CON diet resulted in the highest final weight. The growth and composition of fish can be significantly influenced by the specific ingredients used in organic diets, such as trout remains, Iberian pig viscera, and insects. In the present experiment with European seabass, the lower final fish weights observed in fish fed the IB-IN, IB, and MIX diets could be ascribed to several factors. Firstly, the diet's specific composition, including the inclusion levels and quality of alternative protein sources, may have influenced the growth performance of the fish. It is possible that the proportions or sources of alternative proteins used in these diets were not optimal to meet the nutritional requirements of European seabass. Although the diets were formulated to cover the needs for amino acids, differences in amino acid retention could partially explain these results (Table 5). The amino acid composition of feed, particularly the differential levels of essential amino acids such as methionine and lysine, plays a pivotal role in influencing growth performance. The variations in the levels of these organic amino acids can significantly impact the overall nutritional quality of the diet and subsequently affect the growth outcomes observed in our study. Methionine and lysine are essential amino acids crucial for protein synthesis and various metabolic processes in aquatic organisms. The fact that these amino acids were not present at equal levels in all feeds in our study indicates a potential imbalance in the amino acid profile of the diets. This imbalance can lead to limitations in protein synthesis, affecting the overall growth performance of the aquatic organisms.

Additionally, the observed differences in energy levels may have further contributed to the variations in growth outcomes. Energy is a fundamental factor influencing metabolic processes, and disparities in energy content can affect nutrient utilisation and growth efficiency. The disparities in the levels of essential amino acids, specifically methionine and lysine, coupled with differences in energy levels, likely contributed to the observed variations in growth performance.

Other factors, such as feed palatability, digestibility, and overall diet formulation, including essential nutrients and amino acids, can also play a role in growth performance. In previous studies, the use of insect meal as a partial substitute for fishmeal has shown no negative impact on fish performance in terms of growth, feed utilisation, and digestibility [54–59]. However, these findings contrast with the results of the present study, wherein the IB-IN diet resulted in poorer growth compared to the other experimental diets. This growth deficiency can be attributed to the low digestibility of the IB-IN diet, which may result in a lack of essential amino acids and reduced nutrient availability. In contrast, the TR-IN and IB-TR diets, which incorporated trout meals, resulted in growth approaching that seen with the control diet; the other experimental diets yielded lower growth. Compared to the other experimental diets, the improved growth observed in European seabass fed the TR-IN and IB-TR diets could be explained by the enhanced amino acid profile and improved digestibility. The inclusion of organic alternative fish ingredients such as rainbow trout remains in European seabass diets may offer cost-effective and environmentally sustainable alternatives to traditional fishmeal or plant-based diets. The lowest SGR was found in seabass fed the IB-IN diet, which may be linked to poor energy availability in the IB-IN diet. IN meals can contain a high percentage of chitin, which is composed of glucosamine and a nitrogen-containing substance found in the exoskeletons of insects [60,61]. This protein has complex effects when it is introduced into fish diets from insect-derived sources. Chitin is commonly recognised for its anti-nutritional characteristics and is generally considered unprocessable by fish [62]. In addition to its detrimental influence on nutrient absorption, chitin has been documented to harm the growth rate and feed conversion of tilapia [63].

Body Composition and Nutrient Retention

The present study revealed no significant differences in body composition among European seabass treatment and control groups, including dry matter, crude protein, crude fat, and ash content. The inclusion of alternative organic ingredients, such as trout remains, Iberian pig viscera, and insects did not significantly impact the body composition of the fish. Similar results have been reported in other studies. Gao *et al*. (2020) [64] conducted a study on *Cyprinus carpio* fed blood meal and dried porcine soluble and found no notable variances in moisture, crude protein, and ash composition.Another study by Vélez-Calabria *et al*. (2021) [65] examined the effects of an Iberian pig meal and a vegetable protein blend on seabream. They also reported no significant differences in the protein and ash content of the body among the experimental groups. These findings support the idea that incorporating alternative ingredients into fish diets does not significantly impact body composition in terms of protein and ash content. There is evidence that changes in body composition, especially in body fat, are expected with changes in energy intake. As the intakes in diet were similar, changes in body composition were not expected [66]. These findings showed that incorporating alternative organic ingredients in fish diets, as in the present study, does not result in notable changes in the body composition of European seabass. Nutrient retention and utilisation (PPV and PFV) in the fish were not significantly affected by the inclusion of these ingredients. However, it will be essential to evaluate the long-term effects of these alternative organic diets on body composition and nutrient retention in fish.

The deficit of essential amino acids is a significant challenge when substituting fishmeal with alternative ingredients [67]. The lower retention efficiency of EAA observed in the IB and MIX diets may partially explain the lower final body weights of those fish. This difference can be attributed to imbalances in the amino acid composition of the alternative ingredients. These imbalances can affect the availability and utilisation of essential amino acids, leading to reduced growth and suboptimal muscle efficiency.

The results of the current experiment align with those of previous studies. For example, the TR-IN diet had a similar retention efficiency for Arg (approximately 30%) to that reported by Martínez-Llorens *et al.* (2012) [68]. The high retention efficiency for Arg and His in fish fed the TR-IN and IB-TR diets agrees with the growth results obtained in the study. The results should be interpreted as indicating that these diets provide a favourable amino acid profile and support improved growth performance in European seabass. Arginine and histidine are essential amino acids that play vital roles in various physiological processes, including protein synthesis, immune function, and antioxidant defence.

Digestibility

Before adding ingredients to a commercial aquaculture feed, it is crucial to determine the nutritional quality of each new protein ingredient. The first stage in this process is to assess apparent digestibility and examine how it affects the growth and welfare of different fish species. The results of this study show that trout meal combined with Iberian pig meal (IB-TR diet) and Iberian pig meal (IB diet) improved protein digestibility in European seabass. The variation in protein ADCs among the diets can be attributed to several factors, e.g., the quality and composition of protein sources. Additionally, the trout and Iberian pig meals yielded higher hydrolysis curves and better histidine retention. It is established that specific amino acids and their ratios in the diet can influence protein digestibility and utilisation by fish [69]. Additionally, most of the organic meals used in the experiment were processed from raw sources, with non-standardised methods used to convert them to meal; the processing methods used to produce trout and Iberian pig meals may have influenced their digestibility.

Another study conducted by Tefal *et al.* (2023) [42] reported the highest protein ADC value, 93.0%, for the INS diet. However, insect meal is still far from being a standardised product. In fact, in the present study, the diets containing insect meal (TR-IN and IB-IN) yielded lower protein and energy ADCs, a result that can be attributed to antinutrient factors (ANFs). Insect meal is a potential source of protein and other nutrients, but it contains ANFs that can interfere with nutrient digestion and utilisation in fish [70]. The specific ANFs in insect meals vary depending on insect species, rearing conditions, and processing methods. Some common ANFs in insect meals include chitin, protease inhibitors, lectins, and other bioactive compounds. It should be noted that chitin, a major component of insect exoskeletons, is known for its anti-nutritional properties. It is resistant to enzymatic digestion in fish and can impair the breakdown of proteins and the release of energy from the diet [71].

Fish require phosphorus as a mineral element and component of their bones and scales [72]. Fish must use phosphorus effectively because it can affect feed digestibility, farming costs, and water contamination [73]. In the present work, the ADC of phosphorus for European seabass receiving IB diets was higher than that for fish fed other diets. Fish that ingest various types of byproducts in their diets may experience varied levels of phosphorus availability due to changes in size, particle size of bones, density of bones, processing conditions of fishmeal, or the proportion of non-bone to bone phosphorus in fishmeal [74]. The IB diet had the highest dry matter and phosphorus ADCs, proving that it could be an excellent candidate for inclusion in fish feed and even in organic diets due to its good quality. Its inclusion would probably reduce the need for inorganic phosphate in feed. Supporting the excellent quality of IB protein meal, IB alone (IB diet) and IB in combination with TR meal protein (IB-TR diet) yielded excellent results for protein digestibility compared with the other test diets.

In Vitro Hydrolysis Assay

Simulated digestion experiments are incredibly informative and allow the use of fewer live animals while testing novel feed ingredients with species-specific digestive enzymes [75]. Before investing in expensive in vivo animal-feeding tests, these assays can be helpful tools to evaluate the quality of a prospective feed protein [76]. In vitro digestibility techniques were used in the present study to give a preliminary review of the ability of European seabass to hydrolyse proteins in their diet. The diets investigated could be divided into two groups based on the results of the hydrolysis rate: (a) IB-TR and TR-IN and (b) IB, which showed better results than the rest, especially compared to the control. The results of this study are consistent with the result obtained from the digestibility study in vivo, wherein protein ADC was highest in the IB and IB-TR diets. The times required for complete hydrolysis were 9.24, 9.37, 9.89, and 12.22 h. This result may have significant implications in terms of intestinal transit times and feeding frequencies. Extracts of species-specific enzymes obtained from different sections of the fish digestive system can be used to simulate in vitro digestion. [43].

Other fish species, such as *S. aurata*, *S. senegalensis*, and *salmonids* (mainly rainbow trout and *Oncorhynchus mykiss*), have also been used in the in vitro simulation of fish digestion [43]. The hydrolysis rates obtained with European seabass proteases generally indicate good bioavailability of the proteins in the experimental diet. Similar results were obtained for other ingredients often used in aquatic feeds, such as fishmeal or soybean protein concentrate [77,78]. Other alternative ingredients used in aquafeeds, such as microalgae, have also been found to yield similar results [75]. The existence of alternative organic ingredients in experimental diets with varying rates of protein hydrolysis could have practical relevance in the context of fish nutrition. Highly hydrolysable proteins may rapidly and easily release accessible amino acids, which could stimulate digestion and metabolism. In contrast, the presence of intermediately hydrolysable proteins might lead to a slower rate of amino acid release in the intestinal tract, limiting the saturation of membrane carriers in enterocyte microvilli with amino acids. Importantly, the efficiency of amino acid absorption is influenced by both the relative and total amounts of different amino acids in the intestine.

Discrepancies are frequently observed when comparing crude protein digestibility data obtained through in vivo methods and growth performance. In in vitro hydrolysis assays, as for observations in other animal models, only the bioaccessibility of the protein fraction of the substrate is measured (indicating the susceptibility of this nutrient to enzymatic action). This assessment provides an estimation of potential bioavailability, representing the quantity of amino acids potentially accessible for intestinal absorption. However, this method provides no information regarding the specific amino acids absorbed (essential or non-essential) and the metabolic efficiency in utilizing these amino acids, which is what ultimately influences growth. Consequently, outcomes from in vitro trials offer only partial insights into the metabolic utilisation of proteins. They primarily serve as a tool for selecting protein ingredients based on their total amino acid release under simulated digestive conditions specific to a given species.

Histological Analysis

Understanding the impact of dietary sources on fish pathology requires expertise in animal histology [79]. In general, animal proteins with low levels of antinutritional factors, provided their freshness and quality are satisfactory, tend not to induce liver or intestinal pathologies. Conversely, plant proteins with high levels of these factors are more likely to cause such issues [80].

In our study, feeding with organic byproducts of the Iberian pig, either with or without organic insect meal, appeared to induce liver steatosis in seabass and the fish fed the CON diet. The accumulation of lipid droplets is usually, but not always, associated with a malfunction of metabolism or related to fat metabolism. Other authors have observed steatosis in fish, possibly due to reduced energy availability [81] and liver damage associated with alternative ingredients such as ring-dried blood and feather meals [82]. However, cod fed a combination of wheat gluten and soybean protein concentrate at a maximum supplementation of 44% exhibited no histological alterations in the liver [83].

Conclusions

Diets containing organic alternative raw ingredients yield high growth parameters but result in differences from fish fed the control diet. Most parameters measuring efficiency, such as FCR, PER, PPV, and PFV, show no differences from the control diet, and some parameters, such as apparent digestibility of gross energy or the hydrolysis rates, even yield very promising values. The fish fed the TR-IN and IB-TR diets showed more favourable growth performance than fish fed the IB or IB-IN diet, although slightly lower growth performance than the CON group. This result shows that the organic ingredients used in the diets have potential as more sustainable substitutes for fishmeal. Ultimately, meals derived from byproducts may have significant appeal owing to their cost-competitiveness relative to fish meal, rendering them a potentially attractive and economical choice in organic aquaculture diets.

Acknowledgments

A complete grant from the Ministry of Higher Education of the Arab Republic of Egypt supported the first author. The research project was developed with the collaboration of the Biodiversity Foundation (Spanish Ministry for Ecological Transition and the Demographic Challenge) through the Pleamar Program, co-financed by the European Maritime and Fisheries Fund (EMFF).

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Chapter 4.

Feeding of rainbow trout (*Oncorhynchus mykiss*) with organic ingredients replacing fish meal

Feeding of rainbow trout (*Oncorhynchus mykiss*) with organic ingredients replacing fish meal

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Aquaculture, 2023

Eslam Tefal, David S. Peñaranda, Silvia Martínez-Llorens, Ana Tomás-Vidal, Ignacio Jauralde, Luis Lagos, Francisco Javier Moyano and Miguel Jover-Cerdá, Feeding of rainbow trout (*Oncorhynchus mykiss*) with organic ingredients replacing fish meal, under review in *Aquaculture* 2024, ...

Abstract

Demand for organic aquaculture is rising, but its viability will depend on the availability of economically viable raw materials to formulate organic diets. In the current work, 100% organic diets were formulated based on different alternative protein sources distinguished by their ecological origin, insect meal (IN), seabass byproducts (SB) and Iberian pig byproducts (IB) in rainbow trout (*Oncorhynchus mykiss*) and their effects on growth, efficiency, productivity, and intestinal health. Fish with an initial weight of 67.2 g were fed two times a day until apparent satiation for 150 days. The control diet containing fish meal (FM) originated the highest final weight (298 g). Results obtained in the final body weight and the specific growth rate feed conversion ratio average daily gain indicate that the SB-FM, SB-IB, and SB-IN-IB diets presented a lower performance (272 g, 257 g, and 258 g final weight respectively) and FM-IN and IN-IB diets had the lowest final weight (215g and 183 g respectively). An improvement in growth performance and nutrient utilization was observed in the SB-FM, SB-IB, and SB-IN-IB diets concerning the FM-IN and IN-IB diets. The lowest retention efficiencies of protein, fat, and essential amino acids were found in the IN-IB diet. The highest apparent digestibility coefficients (ADCs) of protein, energy, calcium, and phosphorus are found in Control and FM-IN diets. Results of enzymes showed that both trypsin and chymotrypsin are relatively low in IN-IB and SB-IN-IB. Fish-fed FM-IN and IN-IB diets showed histological changes in the liver and intestine. Considering the intestinal microbiota composition, the three dominant phyla were *Firmicutes (59-89%)*, *Spirochaetota (5-35%)*, and *Proteobacteria (3-16%)*, but no differences between diets were obtained. No significant differences were observed on the Alpha diversity Shannon index. Therefore, although differences in growth were observed, the high substitution of fishmeal did not imply an alteration of the intestinal microbiota, possibly due to the high dominance of *Firmicutes*. Nevertheless, from an economic point of view, SB-IB diets gave the lowest economic conversion index and the highest economic profit index. In conclusion, the substitution of fishmeal affected the growth of the animal, registering the best results in the control followed by diets containing fishmeal of marine origin (SB), but the lowest price of animal byproducts originated the best economic results.

Keywords: Organic trout; fishmeal substitution; organic farm; rainbow trout; microbiota; health status.

Introduction

Ecological production, also called biological or organic, is an agri-food management and production system that combines the best environmental practices, a high level of biodiversity, preservation of natural resources, and the application of demanding standards on animal welfare (FAO, 2020). Mainly, organic aquaculture has grown significantly in recent years, achieving in 2020 6.4% of total EU aquaculture production, around 74.032 tonnes (EUMOFA, 2022b). Therefore, organic aquaculture is a modest but rising part of the worldwide food production industry (Willer *et al.*, 2021b), based on four guiding principles: (1) health, (2) ecological, (3) equity, and (4) welfare (Gould *et al.*, 2019). Most aquaculture production, 82%, is centered in Asia, primarily in China, with the remaining 18% in Europe (Willer *et al.*, 2023).

Trout is the third most abundant aquaculture species, accounting for 4.590 tonnes, mainly distributed as follows: France with 2.346 tonnes, Spain with 917 tonnes, and Denmark with 642 tonnes (EUMOFA, 2022b). Trout is the main species of Spanish continental production, reaching its maximum production peak in 2001 (36,000 tons), but its production was drastically reduced to around 19,400 tonnes in 2020 (APROMAR, 2020). Consequently, organic production may be an excellent option to reinforce trout consumption by providing a differentiated product with added value.

Few studies have been performed about fishmeal substitution for organic diets, being current work the first in rainbow trout. The findings from the research conducted on seabream and seabass indicate that completely substituting fishmeal with organic ingredients, including rainbow trout byproducts, Iberian pig viscera, and insects, brings several benefits in terms of digestibility, histology, and growth performance (Tefal, Jauralde, Martínez-Llorens, *et al.*, 2023; Tefal, Jauralde, Tomás-Vidal, *et al.*, 2023). According to (Lunger *et al.*, 2006, 2007), up to 40% of fishmeal protein can be substituted by NuPro (an organically certified yeast-derived protein source) in juvenile cobia (*Rachycentron canadum*) without harming growth performance. Other studies compared commercial and organic feeds with organic soybean cake and wheat in organic farming of seabass and sea bream, obtaining a similar performance in both diets (Di Marco *et al.*, 2017) without evidence that nutrition affected stress and immunological response.

One of the possible reasons for the few studies performed on organic diet formulation is the need for organic raw ingredients, in addition to reducing the inclusion of raw material from fisheries through fishmeal or oil substitution by alternative sources to achieve sustainable production.

This is a general problem in the aquaculture sector, but organic production is more critical due to the low availability of high-quality alternative sources with an organic origin.

Under organic European Council Regulation (EC, 2008), the diet may contain up to 60% organic plant ingredients and synthetic amino acid addition is prohibited in organic aquaculture diets (EC, 2007). Furthermore, the transitional period for using 30% non-organic fish offal ended in 2014, making the organic diet formulation more complicated. A possible alternative may be the inclusion of Transformed Animal Proteins (TAPs) from non-ruminant animals, whose use is allowed in conventional aquaculture (RD 578/2014), and insects (Reg. EU 893/2017) but currently not explicitly authorized in organic aquaculture. The use of TAPs with organic origin does not imply any contradiction to the regulations, allowing the formulation of organic aquaculture feed without capturing fishmeal, only using the recovery of byproducts resulting from the transformation of organic aquaculture itself or even TAPs from terrestrial organic farms. Organic plant sources are scarce, except soybean, and their amino acid profiles are insufficiently matched to produce an optimal fishmeal. Therefore, considering previous studies of fishmeal substitution in carnivorous species, different alternative protein sources have been investigated: plant-based protein sources, insect meal, TAPs, or organic fish byproducts from the industry. Fishmeal replacement with plant-based protein sources has been widely studied, with the most promising the inclusion of soybean meal (SBM) (Heikkinen *et al*., 2006) or canola protein concentrate (Drew *et al*., 2007), since the addition of soybean meal to the diet, which replaced 45% of fishmeal (FM), led to an increase in feed conversion rate (FCR) and histopathological alterations. The differences in microbiology or inflammation did not impact the animals' performance in trials that lasted up to 18 weeks (Heikkinen *et al*., 2006). Substituting fish oil and fishmeal with vegetable oils and proteins can lower the concentration of polychlorinated dibenzodioxins and polychlorinated dibenzofurans (PCDD/F) and dioxin-like polychlorinated biphenyls (DL-PCB) in rainbow trout and influence their growth rate (Drew *et al*., 2007). Alternative animal proteins have also been used, such as krill, feather meal, meat, bone meal, and bacterial protein; however, mixes of plant-based proteins have produced the best development outcomes (Albrektsen *et al*., 2022; Lee *et al*., 2010). In a study on Atlantic cod, (Toppe *et al*., 2006) utilized fish bone meal (FBM) as a dietary ingredient and study's findings were encouraging, indicating that it may be possible to substitute up to 45% of dietary FM protein with the FBM, which has a crude % protein content of 56%.

Insect meal has been used as a protein source for fish and crustaceans (Mousavi & Zahedinezhad, 2020; Quang *et al*., 2022; Rumbos *et al*., 2021). (St-Hilaire *et al*., 2007) reported that a rainbow trout diet containing 15% black soldier fly prepupae or housefly pupae had no adverse effect on feed conversion efficiency throughout a 9-week feeding period; the average initial weight was 22.6 g.

Aquaculture protein byproducts are a sustainable and cost-effective alternative to conventional fishmeal-based feeds, which can help mitigate the environmental impact of fish processing and reduce the dependence on wild fish stocks for fishmeal production.

Several studies have investigated the use of aquaculture protein byproducts in aquaculture feed. For instance, (S. Li *et al*., 2021) evaluated a combination of shrimp hydrolysate and plant proteins in the diets of largemouth bass, demonstrating that up to 30% of fishmeal can be replaced without any negative effect on growth performance. Similarly, (Gunathilaka *et al*., 2021) studied the use of shrimp protein hydrolysate and krill meal in the diets of red seabream. They found that incorporating shrimp protein hydrolysate can reduce fishmeal usage by up to 20%. Another study by Khieokhajonkhet and Surapon. (2020) assessed the use of fish protein hydrolysate in the diets of Nile tilapia, showed that incorporating 10% resulted in the highest growth performance, feed, and protein utilization. These findings highlight the potential of aquaculture protein byproducts as a valuable ingredient in aquaculture feed formulations.

Summing up, the objective of the current work was to develop for the first time a 100% organic diet for the most relevant European freshwater aquaculture species, rainbow trout, using alternative organic raw materials and evaluate its possible effect on health status through intestinal microbiome composition.

Materials and Methods

Ethics approval

The Committee of Ethics and Animal Welfare of the Universitat Politècnica de València (UPV) reviewed and approved the experimental protocol following the Spanish Royal Decree 53/2013 and the Eu Directive 2010/63/UE on the protection of animals used for scientific purposes.

Experimental setup

The growth assay was conducted at Naturix S.L. (Valderrebollo, Guadalaraja, Spain), a certified organic fish farm for rainbow trout, using 24 cylindrical swimming pools (4 m3) in an open freshwater system. During the experiment, average water parameters were as follows:

temperature of 11±3.3 ºC and oxygen levels of 7.2±1.06 mg L-1. The nitrite, nitrate, and ammonia levels were not detectable during the experiment. All tanks had similar lighting conditions, with a natural photoperiod from July to December.

Fish and acclimatisation

Rainbow trout were provided by Naturix S.L. (Valderrebollo, Guadalaraja, Spain), and acclimatization to pool conditions was necessary. This process lasted two weeks, during which the fish were fed daily to apparent satiation by hand, three times per day (8:00, 13:00, and 18:00) with a control diet (Table 1). A total of 4560 fish were weighed before starting the growth assay (initial weight, 67.2 g) and then randomly distributed into the 24 experimental pools (190 animals/tank).

Diets

Six extruded diets were formulated for the study using organic plant and animal ingredients. The first diet contained fishmeal (FM) as the protein source (Control), while the second diet (FM-IN) used organic insect meal (IN), concretely Hermetia, and fishmeal as the protein source. The third diet (IN-IB) used organic insect meal and Iberian pig byproductsmeal (IB). The fourth diet (SB-FM) used byproducts of organic seabass byproductsmeal and fishmeal as the protein source. The fifth diet used byproducts of organic seabass byproductsmeal and Iberian pig byproductsmeal (SB-IB) as the protein source, and the sixth diet (SB-IN-IB) contained byproducts of both organic seabass and Iberian pig, and insect meal (Table 1). The diets were supplemented with calcium phosphate and vegetable amino acids (lysine and methionine).

Table 1. Ingredients and proximal composition of diets tested in the growth assay.

Ingredients (g kg^{-1})	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**
Raw materials (g kg^{-1})						
Fishmeal[a]	310	100		100		
Insect meal[b]		335	275			160
Seabass by-product[c]				284	293	163
Iberian pork viscera[d]			80		80	50
Wheat[e]	151	112	136	149	144	149
Soybean meal[f]	312	289	309	323	336	309
Wheat gluten[g]	60	60	60	60	60	60

Fish oil	127	59	60	49	32	44
Calcium phosphate	25	15	20	15	25	15
Vegetable methionine[h]	5	10	25	5	10	20
Vegetable lysine[i]		10	25	5	10	20
Vitamins[j]	10	10	10	10	10	10
Nutritional composition[k]						
	86.3	85.0	85.2	89.6	89.2	88.5
CP (% DM)	43.1	43.6	42.5	42.2	41.4	41.7
CF (% DM.)	18.3	18.3	18.1	18.6	19.8	19.1
Ashes (%)	10.2	8.9	8.3	10.2	10.0	8.8
CHO (%)[l]	28.4	29.2	31.1	29	28.8	30.4

[a] Fishmeal (91.9% DM, 73.9% CP, 11.2% CL, 14.3% Ash); CORPESCA S.A.
[b] Insect meal (92.6% DM, 37.6% CP, 28.5% CL, 20% CHO, 13.9% Ash) (Entomoch, Spain)
[c] Seabass byproducts(97.5% DM, 34.5% CP, 41.7% CL, 3% CHO, 20.80% Ash) (Andrómeda, Spain)
[d] Iberian pig byproducts (92.6% DM, 53.0% CP, 28.6% CL, 14.6% CHO, 3.8% Ash) (Jamón y Salud, Spain))
[e] Wheat (92.3% DM, 12.7% CP, 1.3% CL, 1.7% Ash, 19.7 kJ^{-1} Energy); (PIENSOS ecoLUCAT, Barrax, Albacete, Spain)
[f] Soybean meal (94.6% DM, 43.1% CP, 9.3% CL, 6.3% Ash, 19.7 kJ^{-1} Energy); (PIENSOS ecoLUCAT, Barrax, Albacete, Spain)
[g] Wheat gluten (93.7% DM, 87.6% CP, 2.2% CL, 10.2% CHO, 0.05% Ash); (PIENSOS ecoLUCAT, Barrax, Albacete, Spain)

Experimental diets were manufactured as pellets using a semi-industrial twin-screw extruder (CLEXTRAL BC-45, Firminy, St Etienne, France) at the Universitat Politècnica de València. The processing conditions included 100 rpm screw speed, 110°C temperature, 20 atm pressure, and 2-4 mm diameter pellets. The formulation and manufacture of diets were carried out using organic raw materials approved and labelled by Regulation (EU) 2018/848. Once manufactured, the diets were packaged and stored in a thermal insulated tank.

Growth Assay: Nutritional and Biometric Parameters

The trial lasted for 150 days, during which trout were weighed monthly to evaluate their growth and determine nutritional parameters. Throughout the experiment, the fish were fed by hand to apparent satiation three times per day during the first 60 days (at 8:00, 13:00, and 18:00) and twice per day (at 9:00 and 14:00) from then up to the end. The feeding workers distributed the feed slowly, allowing all fish to eat in a weekly regime of feeding days and one fasting day. Every 30 days, all fish were weighed after being previously anesthetized with 10 mg/L clove oil (Guinama®) containing

87% eugenol. Before weighting, the fish were starved for 24 hours. Ten fish were sampled initially at the beginning of the growth trial and stored at -30°C for further whole-body composition analysis. At the end of the experiment, 15 fish per tank were sampled (60 per diet) to assess biometric parameters. Three fish per tank were randomly sampled and pooled to determine proximate composition, fatty acids, and amino acids. Final weight (FW), specific growth rate (SGR), survival, feed intake (FI), and feed conversion ratio, condition factor (CF), viscerosomatic index (VSI), hepatosomatic index (HSI), and meat index (MI) were also measured at the beginning and end of the growth trial. Additionally, the protein and fat retention indexes were calculated to determine their efficiency using the following Equations 1 and 2:

Protein Productive Value[%], PPV = 100 × Protein fish gain [g]/Protein intake[g] (1)

Fat Productive Value [%], FPV = 100 × Fat fish gain [g]/Fat intake [g] (2)

Digestibility assay

The digestibility assay for the experimental diets was conducted at the aquaculture laboratory of Universitat Politècnica de Valencia from February to July 2021 using 300 g rainbow trout specimens from Naturix. Four replicates were carried out for each diet using a square Latin experimental design. The trial was performed in four experimental tanks (190 L fiberglass tanks, 88 cm high, 62 cm wide, and 188 cm deep) set in an open freshwater system based on the Guelph system to collect the faecal material in the settling column. Five fish were placed in each tank. At 10:00 AM, the fish were given one meal daily to prevent waste when the fish were actively feeding. To avoid the pollution of faeces with the diets, the drainpipe and settling column were brushed off an hour after the meal. The following The following morning at 8:00 AM, faeces were gravity-collected from the settling column's base into a plastic container. After collecting faeces, the fish were fed again at 10:00 am, allowing two hours between the activities to reduce stress.

The experiments lasted for 30 days for each diet and each replicate. Prior to analysis, the collected faeces were dried to a consistent weight in a 60°C oven for 48 hours and then stored in airtight plastic containers pending nutrient component and inert marker examination. The apparent digestibility of the diets was estimated indirectly using chromic oxide (Cr_2O_3) (5 g kg^{-1}) as an inert and indigestible marker and measuring its concentration in the diets and faeces. Additionally, dry matter, crude protein, energy, calcium, and phosphorus in both diets and faeces were analyzed using the same method. After acid digestion, the amount of chromium oxide in the diets and faeces was measured using an atomic absorption spectrometer (Perkin Elmer 3300, Perkin Elmer, Boston, MA, USA). Analyses were performed twice. The diets' apparent digestibility coefficients (ADC) were calculated according to (Cho et al., 1982). The ADCs (ADC*dm,* %) dry matter of the diets were calculated using the following Equation 3:

ADCdm % = 1 – (% Cr_2O_3 in diet / % Cr_2O_3 in faeces).

(3)

The following formulas were used to determine the ADCs% of each specific nutritional variable (protein, Energy, calcium, and phosphorus) in the diets:

ADCnut = 1 – ((marker diet/marker faeces) × (nutrient faeces/nutrient diet)). (4)

In this equation, the terms nutrient diet (g kg-1) and nutrient faeces (g kg-1) refer to the nutritional parameters of concern (e.g., protein or energy) in the diet and the faeces, respectively. The terms marker diet (g kg-1) and marker faeces (g kg-1) refer to the marker content of the diet and the faeces, respectively.

Macronutrients and Amino Acids Analysis

Diets and their ingredients, as well as the whole fish, were analyzed according to AOAC, (1990) procedures: dry matter (105°C to constant weight); ash (incinerated at 550°C for 5 hr); crude protein (determined by direct combustion method DUMAS using LECO CN628, Geleen, Netherlands), and crude lipid, (extracted with methyl-ether using ANKOMXT10 Extractor, Macedon, NY, USA). All analyses were performed in triplicate. Diets and whole-body fish amino acids composition (Tables 2 and 3) were analyzed using a Waters HPLC system that included two pumps (Model 515; Waters), an autosampler (Model 717; Waters), a fluorescence detector (Model 474; Waters), and a temperature control module, as described by Bosch *et al.* (2006). After hydrolysis, an internal standard of aminobutyric acid was introduced. AQC (6aminoquinolylNhydroxysuccinimidyl carbamate) was used to derivatize amino acids. After oxidation with performic acid, methionine and cysteine were identified as methionine sulphone and cysteine acid, respectively. Waters AcQ isolated amino acids using a C18 reverse-phase column. 150 mm x 3.9 mm tag. All the analyses were carried out in duplicate.

Table 2. Composition of essential and non-essential amino acids in experimental diets.

Diets	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**
			Essential amino acids (g 100 g^{-1})			
Arginine	3.48	2.40	2.62	2.80	2.56	3.13
Histidine	0.34	0.08	0.62	0.72	0.56	0.64
Isoleucine	1.12	1.40	1.39	1.48	1.36	1.39
Leucine	2.15	2.59	2.73	2.70	2.59	2.56
Lysine	2.81	2.04	2.12	2.52	2.17	2.74
Methionine	0.92	0.88	0.76	0.99	0.87	0.88
Phenylalanine	1.37	1.89	1.89	1.83	1.78	1.72
Threonine	1.57	1.47	1.59	1.60	1.44	1.67
Valine	1.90	2.11	2.13	2.15	2.00	2.12
			Non-essential amino acids (g 100 g^{-1})			
Alanine	2.77	1.63	1.77	1.80	1.64	2.07
Aspartic acid	4.51	3.57	4.25	3.85	4.22	4.98
Cysteine	0.40	0.44	0.45	0.40	0.48	0.69
Glutamic acid	5.69	10.09	9.48	8.39	9.18	6.62
Glycine	3.82	1.64	2.07	1.93	1.83	2.50
Proline	2.28	2.98	2.96	2.34	2.58	1.95
Serine	2.16	1.89	2.58	1.93	2.26	2.58
Tyrosine	1.10	1.42	1.23	1.36	1.19	1.21

FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

Fatty acids analyses

The preparation of fatty acid methyl esters (FAME) from total lipids followed the method described by O’Fallon *et al*. (2007). The analysis of FAME was conducted using a Focus Gas Chromatograph (Thermo, Milan, Italy) equipped with a split/splitless injector and a flame ionization detector. The methyl esters were separated on a fused silica capillary column SPTM 2560 (Supelco, PA, USA) with 100 m × 0.25 mm x 0.2 µm film thickness dimensions. Helium was used as carrier gas with a 20 cm/second linear velocity. The samples were injected with a split ratio of 1/100. The initial oven temperature was set at 140 °C for five minutes, then increased to 240 °C at a rate of 4 °C/minute and held at that temperature for 30 minutes. The detector and injector temperatures were both set at 260 °C. They identified individual fatty acids involved and compared their retention times with standards of fatty acid methyl esters provided by Supelco. Only fatty acids present at a minimum level of 0.1% were considered. To quantify the fatty acids, the sample weight data obtained from the analysis was used to calculate the grams of fatty acids per 100 grams of sample, using C13:0 as the internal standard. The fatty acid composition of the experimental diets is presented in Table 3.

Table 3. Fatty acid composition in experimental diets

Diets	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**
SFA						
(C13:0)	0.37	0.38	0.35	0.30	0.35	0.43
(C14:0)	0.37	1.35	0.33	0.53	0.53	0.89
(C15:0)	0.04	0.07	0.04	0.06	0.05	0.06
(C16:0)	2.66	2.89	3.13	2.78	3.20	3.38
(C17:0)	0.05	0.05	0.05	0.05	0.06	0.06
(C18:0)	0.06	0.04	0.06	0.05	0.05	0.05
MUFA						
(C16:1)	0.42	0.53	0.42	0.60	0.62	0.62
(C17:1)	0.02	0.03	0.03	0.04	0.04	0.03
(C18:1n9c)	4.04	2.86	4.95	4.10	4.91	4.28
(18:1(n-7))	0.38	0.23	0.43	0.43	0.47	0.38
(C20:1)	0.23	0.16	0.01	0.70	0.73	0.39
PUFA						
(C18:2n6c) LA	6.31	2.62	5.76	4.05	4.29	4.26
(C18:3n3) LNA	0.93	0.52	0.79	0.69	0.69	0.60
(C20:4n6) ARA	0.08	0.04	0.19	0.07	0.13	0.12
(20:5n-3) EPA	0.60	0.35	0.45	0.57	0.51	0.46
(22:6n-3) DHA	0.99	0.61	0.68	1.18	1.00	0.77

SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids, LA: Linoleic acid, LNA: Linolenic acid, ARA: Arachidonic acid, EPA: Eicosapentaenoic acid, DHA: Docosahexaenoic acid. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

Blood parameters

Twelve fish per treatment were anesthetized at the end of the study, and blood samples were taken by puncturing the caudal vein with heparinized syringes laced with an anticoagulant. For later analysis, samples were promptly kept at 40 oC. The concentrations of glucose, lactate dehydrogenase (LDH), and total protein (TP) were measured using ultraviolet spectrophotometry (Ortho Clinical Diagnostics, Raritan, NJ, EUA). The ICTIOVET SCP laboratory (Barcelona, Spain) conducted all these analyses.

Enzymatic activity

Three fish's digestive tracts per tank were sampled at the experiment's end. Trout were fed the night before at 20:00 and the day of the sampling at 8:00 to guarantee the presence of content along the whole digestive tract. Fish were sedated with clove oil and slaughtered by cold shock before being dissected to extract the digestive tract. Caeca samples were considered and collected. To prepare for enzymatic extraction, they were kept at 20 °C. Manual disaggregation, dilution in distilled water (1 g of sample: 3 mL of distilled water), homogenization by T 25 digital ULTRA-TURRAX® (IKA®, Staufen, Germany), keeping tubes on ice, and centrifugation at 12,000 rpm and 4 °C for 15 min were used to prepare enzyme extracts for protease analysis. Until enzyme analysis, the supernatant was kept at -20 °C. According to the procedure created by Erlanger *et al*. (1961), trypsin and chymotrypsin activities were obtained by a kinetic test utilizing N-Benzoyl-DL-arginine p-nitroanilide (0.5 mM BAPNA) as a substrate in 50 mM Tris-HCl buffer containing 20 mM CaCl2. Every 30 seconds for five minutes, an increase in absorbance at 405 nm was observed. With an extinction value of 0.0637 mL µg−1 cm−1of p-nitroanilide produced per minute, it was used as the unit of activity. The solubility of protein was determined by the Bradford method (Bradford, 1976).

The enzyme activity of trypsin, chymotrypsin, and the trypsin/chymotrypsin ratio (T/C) are expressed in activity per gram of fish (U g-1 trout), per gram of caeca tissue (U g-1 caeca) and mg of soluble protein (U mg-1 soluble protein)

Liver and Intestinal Histology

At the end of the experiment, intestine and liver samples from three fish from each tank were collected and dissected into small pieces and preserved in formalin 10 %. All 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 and liver examination. A total of 400 sections of the liver and 100 of the intestines were examined under a light microscope (Eclipse E400 Nikon, Izasa S.A., Barcelona, Spain).

The measurements and observations of the intestine were performed using a combination of criteria used in previous studies (Adamidou *et al*., 2009; Nogales-Mérida *et al*., 2016) and the following parameters were measured: serous layer (SL), muscular layer (ML), submucous layer (SML), villi length (VL), villi thickness (VT), and lamina propria length (LP). All the images of samples were taken with an optical microscope Nikon JAPON 0.90. The images were analyzed using Photoshop software and converted into metric units.

Microbiome

At the end of the growth trial, three fish per tank (12 fish per diet) were slaughtered on ice and dissected to obtain the gastrointestinal tract. Fish were fasted for 24 hours before sampling. After discarding the stomach and pyloric caeca, the first intestinal third of the gut (foregut) was dissected, sliced longitudinally, and washed with phosphate-buffered saline solution to remove digestion. Intestinal mucosa was scraped using sterilized large scalpel blades, stored in Eppendorf tubes, frozen in liquid nitrogen, and stored at -80° C. The microbiota of 48 samples collected from posterior rainbow trout intestine were characterized by 16S rRNA gene amplicon sequencing on the Illumina MiSeq platform.

Microbial DNA extraction

According to the manufacturer's instructions, DNA was extracted from 200 L of bacterial suspension using the DNeasy PowerSoil® Kit (Qiagen, Milan, Italy). The samples were lysed in PowerBead Tube using a TissueLyser II (Qiagen) for 2 min at 25 Hz. Like negative control of the extraction procedure, a sample with only lysis buffer was processed in parallel with the samples. The concentration of the extracted DNA was measured with the NanoDropTM 2000 spectrophotometer (Thermo Scientific, Milan, Italy) and stored at 20 C until the PCR reaction was performed.

Preparation of 16S amplicon library and sequencing

Libraries of 16S ribosomal RNA gene amplicons were prepared using primer pair sequences for the V3-V4 region following the Illumina protocol “16S Metagenomic Sequencing Library Preparation” for the Illumina system. Bacterial 16S rRNA gene amplicons were generated from 50 ng of microbial genomic DNA in 25 L PCR using High Fidelity Platinum® Taq

DNA Polymerase Kit (Thermo Fisher Scientific, Italy) and Pro341F (50-CCTACGGGNBGCASCAG -30) and Pro805R (50-GACTACNVGGGTATCTAATCC -30) selected by Takahashi *et al*. (2014). The expected size in the Agilent 2100 bioanalyzer trace after the amplicon PCR step was ~550 bp. The complete procedure for preparing and sequencing the 16S rRNA gene library is described in Rimoldi *et al*. (2018). Briefly, Nextera XT unique reference Illumina paired-end adapters were ligated to 16S amplicons using the Nextera XT Index Kit (Illumina, San Diego, CA, USA). Next, qPCR quality controlled all libraries using KAPA Illumina® Platforms library quantification kits (Kapa Biosystems Ltd., London, UK) at equimolar concentrations and diluted to 6 picomolar. Pooled libraries were then multiplexed and sequenced on an Illumina HiSeq X Ten platform (Illumina, San Diego, CA, USA) at 2x300 bp paired sequences.

Metabarcoding raw data analysis

Raw FASTQ data from sequencing were processed using the open-source program QIIME 2 2021.4 (Bolyen *et al*., 2019). Raw data was quality filtered using the q2-demux plugin, followed by denoising with DADA2(Callahan *et al*., 2016) (via q2-dada2). All amplicon sequence variants (ASVs) were aligned with mafft (Katoh *et al*., 2002) (via q2-alignment) and used to construct the phylogeny with fasttree2 (Price *et al*., 2010) (via q2-phylogeny). Taxonomy was assigned to ASVs using the q2 feature classifier (Bokulich *et al*., 2018) to classify sklearn with its naive Bayes taxonomy V4 (Quast *et al*., 2013). The same analysis was performed after filtering ASV classified as mycoplasmas. In that case, the samples were thinned to 800 sequences per sample.

Statistical analysis

Growth, nutritive, and biometric indices, and all analyses 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 and a level of significance of $p<0.05$. Relative microbiota data were statistically analyzed by one-way analysis of variance using the Newman-Keuls test. Differences were considered statistically significant when $p < 0.05$. The data was expressed as the mean and the standard error of the mean.

3. Results

Growth, nutritive, and biometric assessment

The evolution of fish weight along the growth trial is shown in Fig. 1. During the first 73 days, no differences appeared, but significant differences among diets were observed in the last two samplings.

At the end of the experiment, fish fed the Control diet reached the highest final body weight, followed by fish fed SB-IN-IB and SB-FM diets, while fish fed IN-IB presented the lowest (Table 4).

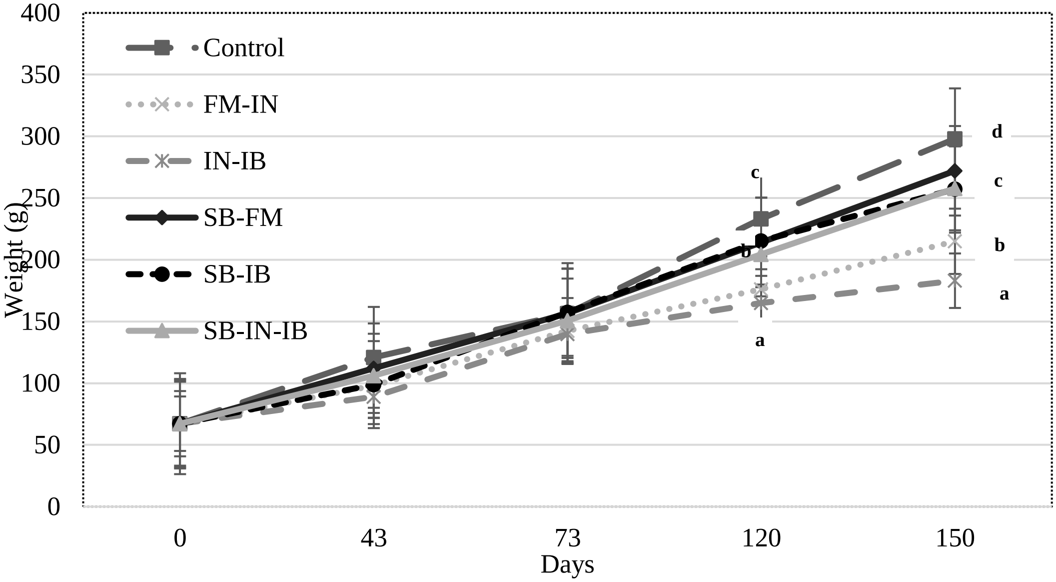

Fig. 1. Evolution of weight along the experiment. Values represented as mean ± standard deviation (n=4). Different letters in each sampling means significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

Fish weight (FW), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), Protein Efficiency ratio (PER), and average daily gain (ADG) were affected by the dietary composition (Table 4). At the end of the experiment, there were no differences among the treatments in fish survival. Trout fed the Control diet with FM presented the highest final weight, SGR, and ADG (298 g, 0.99% day-1, 1.54 g day-1, respectively) and trout fed IN-IB diet had the lowest final weight, SGR, and ADG (183 g, 0.67% day-1, 0.77 g day-1, respectively). FI was the lowest in the FM-IN diet (1.55 g 100 g fish-1 day-1). FCR was the highest in the IN-IB diet (3.37) and the lower in Control diet (2.15) without differences with the other diets (2.14-2.33). PER was the highest in the SB-IB diet and lowest in the IN-IB diet (1.55).

Table 4. Growth and Nutritive indices at the end of the experimental trial

Diets	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**	**SEM**
Final weight	298.0^{d}	215.0^{b}	183.0^{a}	272.1^{c}	257.2^{c}	257.9^{c}	6.6
Survival	78.3	80.5	75.2	69.2	69.7	82.8	5.2
SGR [1]	0.99^{d}	0.77^{b}	0.67^{a}	0.93^{c}	0.89^{c}	0.89^{c}	0.02
ADG[2]	1.54^{d}	0.98^{b}	0.77^{a}	1.37^{c}	1.27^{c}	1.27^{c}	0.04
FI [3]	1.74^{ab}	1.55^{a}	1.91^{b}	1.71^{ab}	1.58^{b}	1.59^{b}	0.11
FCR[4]	2.15^{a}	2.33^{a}	3.37^{b}	2.29^{a}	2.19^{a}	2.14^{a}	0.20
PER[5]	1.14^{ab}	1.11^{ab}	0.84^{a}	1.08^{ab}	1.16^{ab}	1. 21^{b}	0.08

Values represented as mean ± standard error (n=4). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

[1] Specific growth rate (SGR) = 100 × ln (final weight/initial weight)/days.

[2] Average daily gain (ADG) = weight gain (g) / days.

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

[4] Feed conversion ratio (FCR) = feed consumption (g) / weight gain (g).

[5] Protein Efficiency ratio (PER) = biomass gain (g) / protein intake (g)

Body Composition and Retention Efficiency

The proximate composition of the whole body is shown in Table 5. Fish fed the IN-IB diet exhibited the lowest dry matter (22.9 %) and fat content (24.8 %) and consequently the highest body protein (66 %) followed by fish fed FM-IN, whereas fish fed Control and SB-IN-IB diets showed the highest fat (29.9 and 30.0 %) and lowest protein content (59 and 59.4 %, respectively). No significant differences were found for ash content. Differences were observed in Productive Protein Value (PPV) and Productive Fat Value (PFV), which were the lowest values for the IN-IB diet for both (13.7 and 11.7%, respectively).

Table 5. Body composition and retention efficiencies of trout at initial and after feeding with experimental diets.

	Initial	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**	SEM
Dry matter	24.1	25.6^{c}	23.5^{b}	22.9^{a}	25.1^{c}	25.1^{c}	24.8^{c}	0.23
Protein	64.0	59.0^{a}	63.7^{b}	66.0^{c}	61.2^{ab}	61.2^{ab}	59.4^{a}	0.73
Fat	26.8	29.9^{c}	26.1^{ab}	24.8^{a}	27.1^{ab}	28.1^{bc}	30.0^{c}	0.68
Ash	8.4	9.4	10.0	10.0	9.3	9.5	9.5	0.44

PPV [1]	19.9^{b}	18.2^{b}	13.7^{a}	18.7^{b}	20.4^{b}	19.9^{b}	1.30
PFV [2]	25.1^{b}	17.8^{b}	11.7^{a}	19.5^{b}	20.6^{b}	23.8^{b}	1.79

Values represented as mean ± standard error (n=4). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

[1] Productive Protein Value (PPV %) =Protein retained (final fish protein × Final biomass (g)) × 100 - Initial fish protein x initial biomass (g) / Protein ingested (kg ingested food ×% crude protein).

[2] Productive Fat Value (PFV %) =Fat retained (final fish fat × Final biomass (g)) × 100 - Initial fish fat x initial biomass (g) / fat ingested (kg ingested food ×% crude fat).

Regarding biometric parameters (Table 6), statistically significant differences were observed in condition factor (CF), Viscerosamatic Index (VSI) and Hepatosomatic index (HSI). Fish fed FM-IN presented the highest VSI (20.5 %), and fish fed IN-IB diet obtained a lowest HIS and CF (2.0% and 1.0 g cm3-1 respectively). No differences were observed in the Meat index (MI).

Table 6. Biometric indices at the end of the experiment

Diets	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**	**SEM**
VSI[1]	16.78^{a}	20.49^{b}	16.38^{a}	18.10^{a}	17.20^{a}	18.36^{a}	0.54
HSI[2]	2.26^{b}	2.28^{b}	2.00^{a}	2.50^{b}	2.49^{b}	2.27^{b}	0.08
CF[3]	1.20^{b}	1.19^{b}	1.00^{a}	1.31^{b}	1.18^{b}	1.20^{b}	0.03
MI[4]	52.91	47.88	49.33	52.94	48.79	51.03	1.45

Values represented as mean ± standard error (n=12). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

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

[2] Hepatosomatic index (HSI) (%) = (liver weight (g) / total fish weight (g)) × 100.

[3] Condition factor (CF) (g cm_{3}^{-1}) = (total fish weight (g) / length3 (cm)) × 100.

[5] Meat index (MI) (%) = (meat weight (g) / total fish weight (g)) × 100.

Digestibility

The Apparent digestibility coefficients (ADCs) of dry matter for rainbow trout ranged from 78 to 90% without differences among diets (Table 7). ADC of Calcium was the lowest in the SB-IN-IB (23 %) and SB-FM (27 %) diets and highest in the Control diet (65 %). The lowest CDA of phosphorus was for the SB-FM diet (53%), and highest for the Control and FM-IN diets (73 and 74 %). The highest-protein ADCs were observed in fish-fed Control and FM-IN (95 % both), with significant differences in relation to the rest of the diets except the SB-IN-IB diet (87 %). ADCs of energy were the highest in Control and FM-IN diets (93 and 92%, respectively), and the lowest in SB-FM diet (82 %).

Table 7. Apparent digestibility coefficients of dry matter and different nutrients of rainbow trout fed experimental diets.

ADC (%) *	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**	**SEM**
Dry matter	89	90	82	79	84	78	7.12
Calcium	65^{c}	58^{bc}	41^{ab}	27^{a}	41^{ab}	23^{a}	9.88
Phosphorus	73^{c}	74^{c}	58^{ab}	53^{a}	68^{bc}	57^{ab}	6.36
Protein	95^{c}	95^{c}	90^{ab}	87^{a}	90^{ab}	93^{bc}	2.18
Energy	93^{d}	92^{d}	84^{b}	82^{a}	87^{c}	87^{c}	1.12

Values represented as mean ± standard error (n=4). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.
* Apparent digestibility coefficients (ADC).
$ADC_{dm} = 100 - (100 \times (\%\ Cr2O3\ \text{in diet}/\%\ Cr2O3\ \text{in faeces}))$.
ADC_{nut}=100 − (100×(% feed marker/% faeces marker)×(% nutrient. energy. Amino acid. or fatty acid in faeces/% nutrient. energy. amino acid. or fatty acid in faeces).

Retention efficiency of essential amino acids

There were statistical differences in essential amino acid retention efficiency for histidine (His), Isoleucine (Iso), leucine (Leu), and phenylalanine (Phe) (Table 8). Fish fed the Control diet showed the highest retention for His, Iso, Leu, and Phe. Fish fed the IN-IB diet presented the lowest Iso, Leu, and Phe retention efficiency values. The retention efficiency of Arginine (Arg), lysine (Lys), methionine (Met), threonine (Thr), and valine (Val) did not show differences in all diets.

Table 8. Retention efficiency of essential amino acids of the rainbow trout fed with the experimental diets.

Diet	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**	**SEM**
Arginine	13.6	16.6	10.7	17.6	17.6	16.3	1.84
Histidine	53.1^{b}	19.9^{a}	18.8^{a}	25.5^{a}	34.1^{a}	31.7^{a}	5.31
Isoleucine	22.1^{b}	14.0^{ab}	12.1^{a}	16.5^{ab}	17.6^{ab}	17.91^{ab}	1.90
Leucine	21.3^{b}	15.2^{ab}	11.4^{a}	16.9^{ab}	16.6^{ab}	18.6^{ab}	1.85
Lysine	19.4	23.5	19.5	22.6	26.6	20.3	2.25
Methionine	22.5	22.9	22.3	24.4	30.5	24.6	0.02
Phenylalanine	19.2^{c}	12.1^{a}	10.0^{a}	14.4^{ab}	14.8^{ab}	16.3^{ab}	1.62
Threonine	16.3	15.1	10.8	17.3	17.5	16.3	1.58
Valine	18.7	14.9	12.1	16.8	17.8	17.6	1.63

Values represented as mean ± standard error (n=4). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

Enzymatic Activity

Enzymatic activity of rainbow trout fed different experimental diets is shown in Fig. 2. Trypsin activity was lower in fish fed IN-IB and SB-IN-IB diets, but Chymotripsin activity differs in function of units, lowest in SB-FM per g of trout, and lowest in IN-IB in the rest. The ratio Trypsin/Chymotrypsin showed that only was highest in fish fed IN-IB expressed respect to caeca tissue.

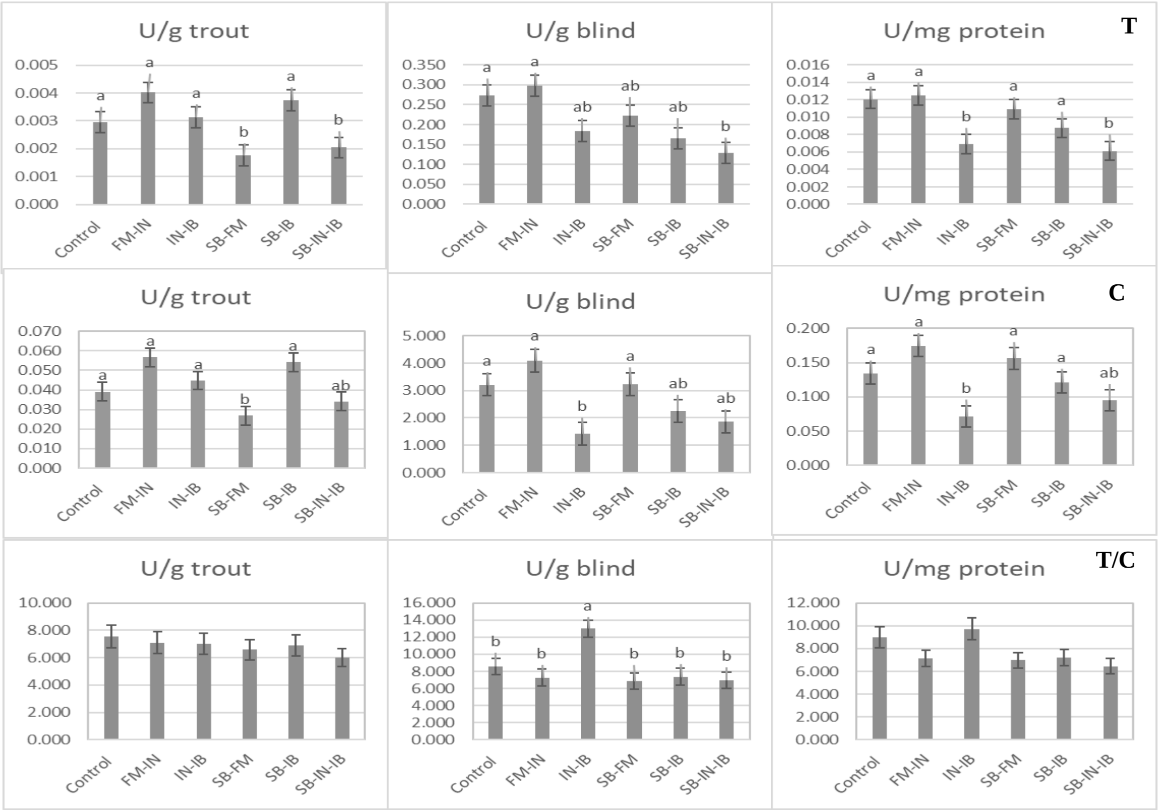

Fig. 2. Activity of digestive enzymes measured in the pyloric cecum of trout fed with experimental diets. Values represented as mean ± standard error (n=4). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic. In the case of (T) trypsin, (C) Chymotrypsin, and the (T/C**)** trypsin/chymotrypsin ratio (T/C), the values for each enzyme are expressed in activity per gram of fish (U g trout^{-1}). Per gram of caeca tissue (U g caeca^{-1}) and mg of soluble protein (U mg soluble protein^{-1}).

Blood Parameters

No differences have been observed regarding parameters analysed in the fish serum fed the experimental diets (Table 9).

Table 9. Effect of the experimental diets on blood parameters of the rainbow trout.

Diets	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**
Glucose (mg/dL)	120.3 ±15.4	147.4 ±9.4	172.9 ±34.7	131.7 ±8.2	130.9 ±17.0	132.0 ±9.3
LDH (U/L)[1]	3694.3 ±491.2	2354.9 ±188.6	2404.3 ±173.9	3826.7 ±687.4	4438.3 ±969.6	3172.8 ±466.0
TP (g/L)[2]	39.9 ±2.1	40.0 ±1.8	36.0 ±1.5	42.3 ±2.0	43.7 ±2.4	43.1 ±2.9

Values represented as mean ± standard error (n=4). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

[1] LDH: Lactate dehydrogenase.

[2] TP: Total protein.

Liver and intestinal histology

Regarding liver histology, some differences have been observed in the nucleus and hepatocytes diameter, as seen in Table 10, which were the highest in fish fed the SB-IB diet (11.16 and 27.58 µm, respectively), whereas they were the lowest (4.78 and 11.67 µm, respectively) in fish fed the FM-IN diet.

Table 10. Histological measures of the liver of trout fed experimental diets.

Diets	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**	**SEM**
Nucleus diameter (µm)	5.77^{c}	4.78^{a}	5.28^{b}	5.92^{c}	11.1^{d}	5.71^{bc}	0.10
Hepatocyte diameter (µm)	14.27^{c}	11.67^{a}	13.17^{b}	12.58^{b}	27.5^{d}	14.06^{c}	0.31

Values represented as mean ± standard error (n=100). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

Differences were found in the qualitative aspect (Fig. 3) between the different diets. Regularly shaped nuclei were observed in the cell's center and peripheral areas. The liver of fish fed with the IN-IB diet showed the absence of white spaces, indicating the accumulation of lipids. The liver of fish fed with Control, IN-FM, and SB-IB diets had a slight fat accumulation. The SB-FM-IB and SB-FM diets showed micro and macro vacuoles with a defined border.

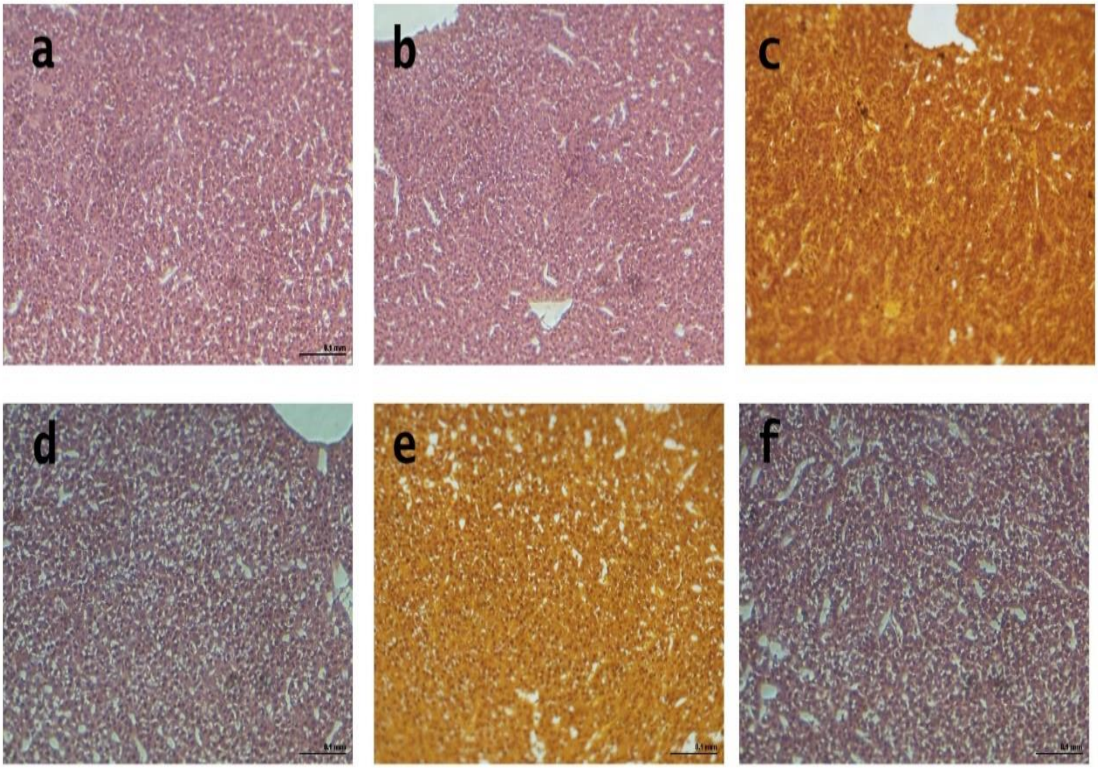

Fig. 3. Histological detail of the liver (20x) with Hematoxylin-Eosin staining, of the trout fed with the experimental diet. (a: FM-IN: Fishmeal-Insect; b: IN-IB: Insect-Iberic; c: SB-FM: Seabass- Fishmeal; d: SB-IB: Seabass-Iberic; e: SB-f: IN-IB: Seabass-Insect-Iberic.

The results of anterior and posterior intestine measurements are reported in Table 11. Significant differences have been observed in all the measurements. The SB-IB diet had the lowest SL, ML, and SML parameters in the proximal and distal intestines (PI, DI), whereas the diet SB-IN-IB registered the highest at PI. LP was the highest for fish-fed SB-IB diet in PI and DI. VL was the lowest for the fish-fed SB-FM diet in PI and DI.

Table 11. Effect of the different diets on proximal and distal intestine measurements in rainbow trout.

	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB	SEM
Proximal							
SL (µm)	59.17^{bc}	56.87^{bc}	41.83^{ab}	73.63^{cd}	27.26^{a}	80.01^{d}	6.85

ML (µm)	78.18^{a}	89.48^{b}	83.46^{ab}	68.39^{a}	67.95^{a}	141.15^{c}	5.06
SML(µm)	46.31^{b}	65.09^{c}	65.82^{c}	47.86^{b}	35.13^{a}	80.99^{d}	2.98
VL (µm)	740.68^{bc}	678.58^{ab}	670.48^{abc}	592.09^{a}	820.63^{c}	812.74^{c}	37.13
VT (µm)	119.76^{a}	118.77^{a}	131.28^{ab}	121.90^{ab}	139.58^{b}	126.79^{ab}	5.92
LP (µm)	$28,22^{b}$	$28,14^{b}$	$24,08^{ab}$	$30,44^{bc}$	$32,83^{c}$	$24,24^{a}$	1,64
Distal							
SL (µm)	123.63^{c}	48.29^{a}	46.22^{a}	82.49^{b}	42.87^{a}	98.57^{b}	9.50
ML (µm)	57.61^{c}	50.67^{ab}	63.57^{bc}	78.53^{c}	37.76^{a}	104.48^{d}	4.98
SML(µm)	56.53^{a}	47.50^{a}	52.58^{a}	55.07^{a}	53.19^{a}	70.50^{b}	4.33
VL (µm)	809.53^{b}	848.41^{ab}	563.01^{ab}	648.85^{a}	656.03^{ab}	802.08^{ab}	82.68
VT 8(µm)	139.85^{b}	108.55^{a}	121.57^{ab}	120.83^{a}	126.30^{ab}	137.68^{b}	5.88
LP (µm)	26.93^{a}	25.55^{a}	24.36^{a}	32.33^{bc}	36.04^{c}	28.02^{ab}	1.89

Values represented as mean ± standard error (n=20). Different letters in the same raw mean significant differences ($p < 0.05$). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic. SL: Serous layer. ML: Muscular layer. SML: Submucous layer. VL: Villi length. VT: Villi thickness. LP: Lamina propria.

Gut microbiota composition

Considering the intestinal microbiota composition, 19 bacterial phyla were found in the sample set (Table 12). Regardless of diet, three dominant phyla were by far the most abundant, Firmicutes (64-81 %). Spirochaetota (8-29 %) and Proteobacteria (3-12), but no difference exists between experimental diets.

Table 12. Phyla found in microbiota sequencing.

Index (%)	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**
Firmicutes	72.57	79.47	77.19	81.07	64.25	78.31
Spirochaetota	14.68	12.07	8.96	8.05	28.68	17.42
Proteobacteria	8.43	5.48	12.03	7.34	4.36	2.64
Actinobacteriota	2.10	1.56	0.51	1.12	1.11	0.97
Bacteroidota	1.29	0.75	0.76	0.73	0.39	0.26
Desulfobacterota	0.04	0.13	0.14	0.43	0.43	0.05
Patescibacteria	0.02	0.01	0.08	0.02	0.00	0.02
Bdellovibrionota	0.10	0.01	0.00	0.01	0.00	0.00
Fusobacteriota	0.04	0.00	0.00	0.18	0.02	0.01
Verrucomicrobiota	0.05	0.01	0.03	0.04	0.01	0.01
Planctomycetota	0.08	0.03	0.02	0.02	0.01	0.01

Acidobacteriota	0.01	0.00	0.00	0.08	0.01	0.07
Deinococcota	0.00	0.00	0.00	0.18	0.02	0.02
Deferribacterota	0.00	0.01	0.01	0.00	0.00	0.00
Myxococcota	0.02	0.01	0.00	0.00	0.00	0.00
Chlorolipids	0.02	0.01	0.00	0.08	0.01	0.00
Gemmatimonadota	0.00	0.01	0.01	0.05	0.01	0.00
Cyanobacteria	0.06	0.02	0.01	0.01	0.00	0.01
Methylomirabilota	0.00	0.00	0.00	0.03	0.04	0.00
Unassigned	0.50	0.41	0.24	0.57	0.65	0.18

Values represented as mean (n=6). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

A number of 27 Genera were found in the microbiome. The dominant genera of microbiota were Mycoplasma (59-88 %) within the Firmicutes phylum, followed by Brevinema (5-35 %) (Table 13), but without statistical differences between diets. Clostridium and Xanthomonas were higher in IN-IB than in the rest of the diets but without significant differences.

Table 13. Taxonomy and percentage (%) of the bacterial genera detected in the microbiota of hindgut samples (for each experimental group).

Index (%)	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**
Mycoplasma (Firmicutes)	70,51	77,73	72,90	78,55	57,67	88,12
Brevinema (Spirochaetota)	14,68	16,86	5,41	8,85	34,78	7,87
Sphingomona (Proteobacteria)	0,58	0,76	0,51	1,55	0,69	0,34
Clostridium (Firmicutes)	0,00	0,00	3,19	0,19	0,03	0,01
Xanthomonas (Proteobacteria)	0,34	0,00	10,06	0,00	0,11	0,00
Blastomonas (Proteobacteria)	1,74	0,17	1,23	0,00	0,01	0,00
Crenobacter (Proteobacteria)	0,00	0,86	0,07	0,52	0,62	0,05
Aeromonas (Proteobacteria)	0,26	0,08	0,05	0,76	0,10	0,48
Corynebacterium (Actinobacteriota)	0,89	0,02	0,01	0,22	0,48	0,10

Streptococcus (Firmicutes)	0,86	0,01	0,02	0,23	0,04	0,03
Aquabacterium (Proteobacteria)	1,20	0,00	0,00	0,00	0,00	0,00
Acinetobacter (Proteobacteria)	0,08	0,03	0,03	0,65	0,00	0,03
Xanthobacteraceae (Proteobacteria)	0,18	0,00	0,01	0,24	0,07	0,29
Amaricoccus (Proteobacteria)	0,00	0,00	0,00	0,34	0,00	0,36
Bacteroides (Bacteroidota)	0,05	0,17	0,17	0,03	0,25	0,13
Massilia (Proteobacteria)	0,03	0,00	0,01	0,69	0,00	0,02
Mycobacterium (Actinobacteriota)	0,09	0,01	0,21	0,14	0,24	0,08
Staphylococcus (Firmicutes)	0,12	0,10	0,03	0,01	0,50	0,02
Bosea Proteobacteria)	0,51	0,02	0,46	0,00	0,00	0,00
Desulfovibrio (Desulfobacterota)	0,02	0,02	0,04	0,51	0,07	0,01
Escherichia- Shigella (Proteobacteria)	0,01	0,00	0,42	0,07	0,05	0,05
Clostridiaceae (Firmicutes)	0,25	0,00	0,00	0,02	0,13	0,00
Hydrogenophaga (Proteobacteria)	0,27	0,03	0,45	0,02	0,00	0,00
Brevundimonas (Proteobacteria)	0,15	0,00	0,00	0,06	0,01	0,23
Roseococcus (Proteobacteria)	0,35	0,05	0,36	0,00	0,00	0,00
Flavobacterium (Bacteroidota)	0,21	0,00	0,09	0,11	0,05	0,01
Deefgea (Proteobacteria)	0,03	0,02	0,03	0,04	0,44	0,00

Values represented as mean (n=6). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

Mycoplasma was genera predominant within the Firmicutes phylum without significant differences between groups, and due to the dominant character of the Firmicutes phylum and the Mycoplasma genera, the sequences were filtered for these genera since it could be camouflaging possible. After filtering, the dominant phylum (Table 14) was, Spirochaetota (27-54 %) and Proteobacteria (18-37 %).

Table 14. Taxonomy and percentage (%) of the bacterial phyla detected in the microbiota of hindgut samples after Mycoplasma filtration.

Index (%)	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**
Spirochaetota	27,32	52,09	30,44	36,39	54,15	37,79
Proteobacteria	43,74	18,09	37,48	26,98	21,37	28,35
Firmicutes	7,15	13,12	15,06	8,47	8,95	10,87
Actinobacteriota	7,43	2,90	4,93	9,36	6,36	9,09
Bacteroidota	5,61	5,27	4,33	3,85	1,25	5,76
Patescibacteria	0,04	0,05	0,33	0,04	0,00	0,27
Desulfobacterota	0,07	2,12	1,32	3,29	1,18	0,45
Fusobacteriota	0,00	0,16	0,02	0,64	0,11	0,13
Planctomycetota	0,13	0,06	0,10	0,11	0,17	0,10
Bdellovibrionota	0,17	0,00	0,00	0,05	0,00	0,03
Verrucomicrobiota	0,08	0,14	0,44	0,10	0,01	0,00
Methylomirabilota	0,00	0,00	0,00	0,29	0,00	0,00
Deinococcota	0,00	0,01	0,00	0,84	0,04	0,14
Chloroflexi	2,86	0,00	0,00	0,32	0,00	0,00
Acidobacteriota	0,02	0,00	0,05	0,26	0,00	0,68
Gemmatimonadota	0,00	0,19	0,02	0,18	0,00	0,00
Cyanobacteria	0,05	0,13	0,03	0,02	0,00	0,14
Myxococcota	0,03	0,09	0,25	0,00	0,00	0,00
Deferribacterota	0,00	0,13	0,02	0,00	0,00	0,00
Not assigned	5,30	5,45	5,19	8,82	6,41	6,18

Values represented as mean (n=6). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

After filtering, the most dominant genera in the Spirochaetota phylum was Brevinema as the most abundant genera in all the experimental diets, with a higher percentage in the SB-IB and FM-IN diets (54.2 ± 11.5% and 52.1 ± 12.5%, respectively (Table 15).

The Proteobacteria phylum was mainly represented by the classes Alphaproteobacteria and Gammaproteobacteria. The Alphaproteobacteria class had a differential representation according to the diet, with the genera Sphingomonas, Roseobacter, and Brevundimonas being more abundant in the "SB-IB" diet. On the other hand, the "IN-IB" diet had a higher representation of Blastomonas and the "Control" of Bosea. In the class Gammaproteobacteria, the principal genera found were Dechloromonas and Thermomonas in the "IN-IB" diet, Hydrogenophaga in the "SB-FM" diet, and Shewanella in the "FM-IN" diet. The third most abundant phylum was Firmicutes, with 8.98% of the total.

Table 15. Taxonomy and percentage (%) of the bacterial genera detected in the microbiota of hindgut samples after Mycoplasma filtration (for each experimental group).

Index (%)	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**
Brevinema (Spirochaetota)	28.9	52.1	27.3	36.4	54.2	35.5
Sphingomonas (Proteobacteria)	17.6	2.8	5.7	6.4	5.2	35.5
Clostridium (Firmicutes)	0.0	0.0	5.0	0.5	0.2	0.1
Xanthomonas (Proteobacteria)	1.0	0.0	10.1	0.0	0.8	0.0
Blastomonas (Proteobacteria)	3.2	1.7	1.2	0.0	0.1	0.0
Crenobacter (Proteobacteria)	0.0	4.1	0.1	1.0	2.4	1.1
Aeromonas (Proteobacteria)	0.4	1.2	0.1	4.5	0.1	3.8
Corynebacterium (Actinobacteriota)	3.3	0.6	0.1	3.4	2.8	3.2
Streptococcus (Firmicutes)	0.0	3.7	0.8	1.0	0.2	0.3
Aquabacterium (Proteobacteria)	0.0	0.0	0.0	0.0	0.0	0.0
Acinetobacter (Proteobacteria)	0.0	0.2	1.4	1.8	0.0	0.3
Xanthobacteraceae (Proteobacteria)	1.1	0.0	0.3	1.0	0.3	2.4
Amaricoccus (Proteobacteria)	0.0	0.0	0.0	0.9	0.0	3.7
Bacteroides (Bacteroidota)	0.8	1.6	2.8	0.8	0.3	1.1
Massilia (Proteobacteria)	0.0	0.0	0.1	2.1	0.0	0.4
Mycobacterium (Actinobacteriota)	1.7	0.1	2.1	0.8	0.9	0.8
Sthapylococcus (Firmicutes)	0.4	0.7	0.5	0.0	3.0	0.6

Bosea (Proteobacteria)	0.9	0.2	0.5	0.0	0.0	0.0
Desulfovibrio (Desulfobacterota)	1.2	0.0	0.0	0.0	0.0	0.2
Escherichia-Shigella (Proteobacteria)	1.9	0.0	6.7	0.2	0.4	1.3
Other genera	37.5	31.0	35.3	39.0	29.1	9.7
Lactobacillus (Firmicutes)	0.00	0.28	2.10	0.46	0.34	0.93
Flavobacterium (Bacteroidota)	0.56	0.00	0.13	0.60	0.33	0.07

Values represented as mean (n=6). Test Newman-Keuls. FM-IN: Fishmeal-Insect; IN-IB: Insect-Iberic; SB-FM: Seabass- Fishmeal; SB-IB: Seabass-Iberic; SB-IN-IB: Seabass-Insect-Iberic.

Despite genera differences in the different diets, when diversity was assessed using the Shannon index, there were no significant differences (Fig. 4).

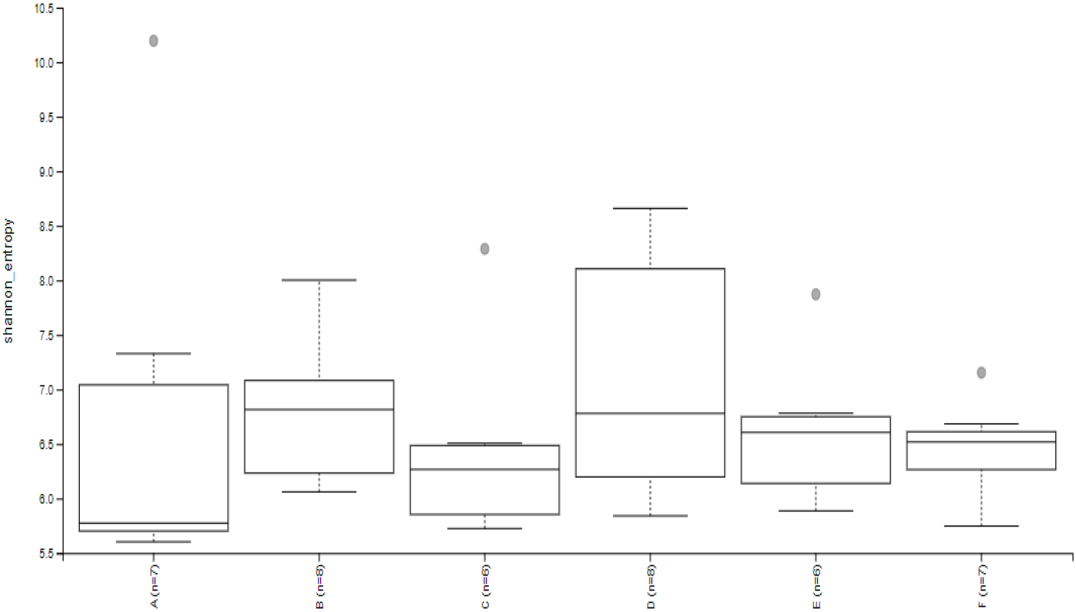

Fig. 4. Representation of diversity through the Shannon index between the different experimental groups.

(A) Control, (B) FM-IN: Fishmeal-Insect; (C) IN-IB: Insect-Iberic;(D) SB-FM: Seabass- Fishmeal; (E) SB-IB: Seabass-Iberic; (F) SB-f: IN-IB: Seabass-Insect-Iberic.

4. Discussion

Herbivorous and omnivorous species from organic production, as tilapia and catfish, seem to be easier than carnivorous species since organic feedstuff can cover their nutritional needs and thus easily replace conventional feedstuff (Craig and McLean, 2005). Nevertheless, formulating organic diets for carnivores is much more difficult due to their high protein/essential amino acid requirement and the prohibition of organic terrestrial animal byproducts and synthetic amino acids in organic diets (Unión Europea, 2018). Nevertheless, the results of the present work are promising.

Fish Performance and Biometric Parameters

No significant differences in mortality were observed among the experimental diets, ranging from 17% to 30%, with a concentrated increase between July 28th and August 2nd due to a flow-related issue.

Good results have been obtained in trout using diets with high substitutions of fishmeal for vegetable mixtures in conventional feeds (Burr et al., 2012; Watanabe et al., 1993), but there are no studies where growth is evaluated in trout fed with organic feed with high substitutions of fishmeal and fish oil. Some previous works with seabass compare conventional and organic diets. However, the results cannot be well compared since the organic diets had higher amounts of fishmeal (56%) than the conventional one (20%), resulting in better growth and feed conversion ratios in fish-fed organic feed. (Di Marco et al., 2017). In other similar studies carried out with sea bream, the organic feed also presented better growth than the conventional one, without significant differences, but the organic feed was also formulated with a higher percentage of fishmeal (63%) than the conventional one (50%) (Mente et al., 2012). Two studies evaluated the effect of organic raw materials such as organic insect meal, Iberian pig byproducts, and organic rainbow trout byproducts for gilthead seabream; total replacing fishmeal with organic raw materials provides numerous advantages in terms of digestibility, histology, and growth performance (Tefal et al., 2023b, 2023c). In another investigation with the same ingredients for seabass carried out by Tefal et al. (2023a), it was found that the complete substitution of fishmeal slightly impacted growth and certain efficiency parameters, although not significant enough to outweigh the economic benefits.

The control diet with fish meal as animal protein gave the best results, but the two experimental diets containing organic insect meal (IN-IB and FM-IN) obtained the lowest final weights, indicating a negative effect on the growth performance of rainbow trout. The poor growth observed might be attributed to the low dietary level of marine proteins (considering both, fishmeal and seabass by-products) in these diets (10 and 0%, respectively, for IN-IB and FM-IN) when they are compared with the rest of the diets (38,4% in the SB-IB diet, 31% in the Control diet, and 16% in SB-IN-IB diet). Therefore, the inclusion of alternative marine sources (organic seabass by-products) reversed the negative effect on fish growth, and it may be a more economical and environmentally sustainable option than only fishmeal or plant-based diets.

Likewise, the fish fed with the IN-IB diet had the worst FCR and poorest both protein and lipid retention, which may indicate a nutrient unbalance (Goff and Gatlin, 2004). On the other hand, the SB-IN-IB diet containing a lower percentage of insect meal (16% insect meal) was closer to the results obtained with the Control diet in nutritive parameters, particularly FCR. In previous studies, insect meal has partially or totally replaced fishmeal (50% and 45%) without affecting fish growth performance, feed utilization, digestibility, microbiota, and fillet quality (Iaconisi et al., 2017; Magalhães et al., 2017; Rimoldi et al., 2021; Terova et al., 2021). However, as in the current work, growth was affected when this substitution was 50 % (Melenchón et al., 2022). Insects were generally high in fat (20 %) compared to fishmeal (Domínguez, 2015); the fatty acid profile of the diets with insects (IN-IB and FM-IN) had a higher ratio of saturated fatty acids, which differs from that of fishmeal (Control), which is rich in n-3, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), lowest in insect diets (Table 3). Fish oil in insect diets had to be reduced to maintain the lipid level, but diets containing organic seabass byproducts (42 % lipid) did not need fish oil. EPA and DHA were higher than in insect diets, and growth was higher, although lower than Control diet. The differences in CF and HSI in this study were caused by the smaller size of the IN-IB fish and poor efficiency, which resulted in small size fish.

Body Composition, Retention Efficiency, and Amino Acids Retention

Concerning body composition, dry matter, and fat composition showed significant decreases when the fish were fed FM-IN and IN-IB diet, probably due to poor growth. Nevertheless, diet SB-IN-IB, containing a lower-level insect meal, showed a similar body profile to the control diet.

The current study's findings show that substituting fishmeal affects the apparent retention values of many essential amino acids (EAA), which is one of the most severe issues with FM substitution with alternative ingredients is an essential amino acid deficiency (Kaushik and Seiliez, 2010), and unbalanced EAA levels in diets have been identified as a significant cause of low growth in the fish-fed animal by-products -based diets (Moutinho et al., 2017).

Control diets had high percentages of some EAA retention efficiency, and their values are comparable to previous works, but no differences appeared in arginine, lysine, and methionine retention (Moutinho et al., 2017). According to the growth results obtained with this diet, the fish fed with the IN-IB diet had the lowest retention efficiency for some EAA, histidine, isoleucine, leucine, and phenylalanine.

Enzymatic Activity and Digestibility

In the present study, the results of the enzymatic activity showed, in general, that the rainbow trout fed Control, IN-FM, and SB-FM diets increased the trypsin and chymotrypsin activity of the pyloric caeca (**Fig. 3**). On the contrary, fish fed with IN-IB diet showed a general decrease in trypsin and chymotrypsin activity. Since digestive enzymes are a useful indicator of feed digestion in fish, the characterization provides information on the digestive ability of fish to hydrolyse proteins in feed ingredients (Lemieux et al., 1999). Therefore, the low digestive capacity observed in the IN-IB diet is directly correlated with low growth and low protein and lipid retention efficiency, and poor digestibility obtained for this treatment, as shown in Table 3**,** Table 4, and Table 6, respectively.

In the present study, diets with a high fishmeal content demonstrated higher digestibility. The increased digestibility of dry matter, energy, nitrogen, and amino acid availability reaffirms the preference for fishmeal as the primary protein source in formulated aquaculture feeds. Previous research on salmonids has also supported the high digestibility coefficients observed (Smith and Guerin, 1995; Sugiura et al., 1998). Similarly, other ingredients, such as animal meals and protein extracts, such as gluten from corn and wheat, exhibit comparable digestibility to fishmeal in silver perch (Geoff et al., 2001). Therefore, it is evident from the current study that the Control diet, with the highest fishmeal content, resulted in enhanced digestibility.

Indeed, the apparent digestibility coefficients (ADC) of Crude protein, Energy, Calcium, and Phosphorus significantly decreased when the fish were fed a diet of SB-FM.

Blood Parameters

All the blood parameters observed are within the normal range of those established for this species (Carthy et al., 1971). No differences have been observed in the parameters analysed in the serum of 12 fish per treatment.

Histological analysis

liver

Significant differences were found in the liver histology of fish fed the experimental diets. Fish fed the SB-IB diet exhibited the highest nucleus and hepatocyte diameter measurements, whereas those fed the FM-IN diet demonstrated the lowest measurements for both parameters. This work indicates that the FM-IN experimental diet had a lower content of highly unsaturated fatty acids (HUFAs) than the Control diet. This finding is significant because it suggests that the substitution of certain ingredients in the experimental diets resulted in a decrease in the levels of essential fatty acids. According to numerous studies, these observations are consistent with the fact that high levels of substitution cause an increase in fat in the liver, increasing the hepatosomatic index, and the content of lipid vesicles in carnivorous fish (Jerusalén, 2017). Some authors have related that the reduction of the content of essential fatty acids in the diet tended to cause deposition of lipids in the liver since it is known that low levels of n3 HUFAs in the diets produce a decrease in the synthesis of lipoproteins, preventing the transport of lipids from the liver to other tissues (Cansino, 2002). This suggests that the altered fatty acid composition in the experimental diets may have contributed to the observed changes in liver histology. After analysing the measurements obtained in each treatment, a relationship was observed between the final weight, the diameter of hepatocytes, and nuclei in the liver Despite the established differences, no significant pathological alterations of the liver tissue were observed because of replacing the fishmeal.

Intestine

In the present study, the histological sections of the foregut showed typical morphologies of a rainbow trout were observed under normal conditions, except in the IN-IB diet that presented thickening, and a reduction of the intestinal villi height may be due to the accumulation of fluid and infiltration of inflammatory cells (Estruch et al., 2018). The SB-IN-IB diet was found with higher measurements of ML and SML than the rest, but without morphological alterations and with optimal growth. The Control, SB-FM, and SB-IB diets coincide with the most efficient diets regarding growth. The values obtained in the hindgut of rainbow trout did not show differences as evident as in the case of the foregut between the different treatments. However, the SB-IN-IB diet followed by the Control diet have been the diets that have recorded the greatest lengths and thicknesses. As in the foregut, in villi lengths the influence of fishmeal on intestinal morphology has once again been detected, where treatments without fishmeal tended to shorten villi length. In a previous study, the experimental group without fish meal (FM0) had higher VT and LP values at PI; however, the opposite trend appears to be observed at DI, with lower VT and LP values but no significant differences (Vélez-Calabria et al., 2021). The IN-IB diet's observed morphological alterations, such as smaller measurements and decreased absorption surface, could have contributed to its lower growth efficiency. Similar findings have been reported in previous studies. (Santigosa et al., 2008) investigated the replacement of fishmeal with vegetable raw materials in rainbow trout and found that as the fishmeal content decreased, there was a decreasing trend in villi length and smaller goblet cells, indicating potential negative effects on intestinal morphology. Furthermore, another study focused on replacing fishmeal with insect meal and found that the control diet with fishmeal had greater thicknesses, suggesting a positive impact on intestinal morphology (Melenchón et al., 2022). These findings support the idea that alterations in diet composition, particularly the substitution of fishmeal with other ingredients, can influence the morphological characteristics of the intestine. The observed smaller measures and alterations in villi length and goblet cells in the IN-IB diet may have contributed to the lower growth efficiency observed in the study.

Microbiome Analysis

The microbial communities that inhabit the gastrointestinal tract of vertebrates are closely connected to their digestive physiology and gut health (Lyons et al., 2017). We can conclude that regardless of diet, the most dominant phylum by far in the present study was *Firmicutes*, with a mean of 76.02%. *Mycoplasma* is the main genera in all of them. These data largely agree with those shown by, where the phylum *Firmicutes*, *Proteobacteria*, and *Tenericutes* were dominant in the intestine of rainbow trout, regardless of diet (Lyons et al., 2017; Terova et al., 2019).

Mycoplasmas are bacteria that lack a cell wall; they are one of the smallest organisms capable of self-replication. The small size of the genome of *Mycoplasma*. It is presumed to be the result of close interaction with its host, which has resulted in the loss of part of its genome (Rasmussen et al., 2021; Razin, 1992). Most host-associated *Mycoplasma* genomes are smaller than 1 Mb and contain less than 1000 protein-coding genes. *Mycoplasmas* are recognized as parasitic or commensal with their host. They have undergone reduction evolution from the *Bacillus/Clostridium* branch of Gram-positive eubacteria, often resulting in a reduction in the number of genes in the genome (Dandekar et al., 2002).

Although the genera *Mycoplasma* are often exposed as an obligate parasite, studies have revealed that *Mycoplasma* species, as a natural host in salmonids, could be adapted explicitly for ammonotelic hosts as most teleosts, due to the ability to utilize ammonia. In the intestine. It is hypothesized that this could have facilitated a beneficial evolutionary relationship between *Mycoplasma* and its salmonid hosts (Rasmussen et al., 2021).

Studies of the gut microbiome in salmonids showed *Mycoplasma* as the predominant genera. These salmonid-related *Mycoplasma* species are highly dominant in the gastrointestinal microbiota of all salmonids investigated, including rainbow trout (*Oncorhynchus mykiss*), Chinook salmon (*Oncorhynchus tshawytscha*), and Atlantic salmon (*Salmo salar*). Furthermore, phenotypic evidence points towards a beneficial role of *Mycoplasma*, such as disease resistance, given the inverse correlation between the abundance of *Mycoplasma* and *Vibrio sp.* (Brown et al., 2019; Lyons et al., 2017; Rasmussen et al., 2021).

Previous studies have shown the importance of arginine and its derivatives, citrulline and ornithine, in the gastrointestinal tract of farmed fish. There is genetic evidence that *Mycoplasma* can use ammonia as a substrate for ornithine and citrulline biosynthesis due to the presence of genes encoding carbamate kinase (arcC) and ornithine transcarbamylase (otc), becoming an important power source (Andersen et al., 2013; Berge et al., 2002; Nguyen et al., 2018; Wang et al., 2020). This characteristic benefits salmonids since they cannot synthesize arginine de novo. In addition, it could increase the detoxification of ammonia in the intestine, which is usually found in high concentrations. On the other hand, ornithine absorption from the intestine may lead to increased growth in Atlantic salmon (Li et al., 2009; Rubino et al., 2014)

Finally, *Mycoplasma* in salmonids harbor genes capable of degrading long-chain polymers, such as chitin, which is usually abundant in insects and crustaceans, which constitute a significant proportion of the natural diet of juvenile salmonids. This could be beneficial for its host, as degradation of long-chain polymers increases the nutritional value of a chitin-rich diet and thus could be a coevolutionary driver between salmonid and *Mycoplasma* hosts. This hypothesis may also explain the increase in *Mycoplasma* in aquaculture cohorts, where an increase in *Mycoplasma* was shown in the intestinal region of rainbow trout reared on an insect-based diet, which has subsequently been shown to be beneficial (Orlov et al., 2006; Rimoldi et al., 2021, 2019). Furthermore, chitin and its deacetylate derivative, chitosan, have antimicrobial properties and a bacteriostatic effect against various harmful gram-negative bacteria (Nawaz et al., 2018).

Once *Mycoplasma* filtered the data, *Firmicutes* decreased from high percentages to significantly lower values. The phylum that increased the most was *Spirochaetota*, with the genera *Brevinema* being the only representative, specially important in trout fed SB-IB diet, a genus associated with more excellent resistance to diseases (Mora-Sánchez et al., 2020). *Brevinema* is part of the central microbiota of Atlantic salmon (*Salmo salar*). It is associated with the expression of genes related to proinflammatory and anti-inflammatory responses. The *Spirochaetota* phylum has been associated with the expression of genes related to intestinal barrier function. Nevertheless, the *Proteobacteria* Phylum was more abundant than *Spirochaetota* in the control diet. Other genera, such as *Clostridium* and *Xanthomonas*, were highest in the trout-fed diet IN-IB, which had the worst growth results, and *Blastomonas* and *Aquabacterium* were the highest in the Control diet.

On the other hand, it is well documented that rainbow trout misuse dietary carbohydrates (Geurden et al., 2014; Guillaume et al., 2001), but the cause remains unclear (Lyons et al., 2017). Members of the phylum *Firmicutes* and *Spirochaetota* are known to play essential roles in the fermentation of dietary carbohydrates, transporting indigestible sugars across their cell membranes (Corrigan et al., 2015; Lyons et al., 2017). For most microbial fermentations, glucose dissimilation occurs via the glycolytic pathway. The molecule most frequently produced from this process is pyruvate. Therefore, the elevation of the glycolysis/gluconeogenesis and pyruvate metabolism pathways represents a further indication of the fermentative potential of the trout gut microbiome. This may be correlated with *Firmicutes* as one of the significant microbial phyla observed in the intestine of rainbow trout. Carbohydrate fermentation results in the formation of short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, which can be used in energy metabolism and have also been shown to promote enterocyte health (Hamer et al., 2008; Louis and Flint, 2009). Furthermore, high SCFA concentrations have previously been reported in various fish species, including rainbow trout (Clements et al., 2014; Lyons et al., 2017; Smith and Guerin, 1995).

The elevation of the genetic pathways responsible for the fermentation of amino acids and the production of peptidases could be related to the protein richness of the food. Rainbow trout require high levels of dietary protein, more than 35% of dietary dry matter, most likely associated with persistent amino acid catabolism for use as an energy source (Geurden et al., 2014; Kaushik and Seiliez, 2010).

Dietary proteins not digested by endogenous digestive enzymes are made available to bacteria for fermentation. Thus, microbiome fermentative activity may be significant in the distal intestinal region, where such enzymes are likely to have less influence (Lyons et al., 2017). On the other hand, in the *Firmicutes* phylum, there are *clostridia* with proteolytic and amino acid fermenting capacity (Neis et al., 2015). This could be an advantage for the group fed with the "FM-IN" diet since *Clostridium* was represented in a higher proportion.

Regarding the *Lactobacillus* genera, the proliferation of lactic acid bacteria

(LAB) may be due to the prebiotic effect of chitin and, as Bruni et al. (2018), may indicate that chitin was a growth substrate for BAL. The group fed with SB-IN-IB diet was the one that obtained the highest proportion of *Lactobacillus*, being the most numerous genera of *Firmicutes* for this diet after *Mycoplasma*. These data do not agree with Bruni et al. (2018) results, since this group is not the one with the highest percentage of insects in its composition. In the remaining plots, this genus is found in a smaller proportion. *Lactobacillus* play an essential role in fiber degradation. In addition, they have an active role in the host's defence against pathogens by producing bactericidal compounds, such as lactic acid, hydrogen peroxide, bacteriocins, and biosurfactants, which prevent pathogen colonization of the intestinal epithelial surface (Corr et al., 2007; Rimoldi et al., 2021; Ringø et al., 2018).

Economic analysis

Although trout growth using organic ingredients was reduced, this work has demonstrated that trout can grow and allow commercial weight with organic byproducts from seabass and Iberian pork, with a similar feed conversion ratio. On the other hand, the growth and conversion ratio of experimental diets SB-FM, SB-IB, and SB-IN-IB was the same than using the commercial diet of the fish farm, (286 g and 1.9, data not showed), which opens an opportunity for cheaper organic diets, because the lower growth could be compensated by lower cost of diets (Table 16).

Table 16. cost and economic performance metrics for experimental diets in rainbow trout.

	Control	FM-IN	IN-IB	SB-FM	SB-IB	SB-IN-IB
Price of diet (€/kg)	1.86	3.34	2.95	1.38	1.26	2.21
Economic Conversion(€/kg)	4.00	7.78	9.96	3.15	2.77	4.73
Profit Index (€/kg)	4.00	0.22	-1.96	4.85	5.23	3.27

The price and economic conversion were lowest with diets SB-FM and SB-IB, and consequently, the Profit Index, expressed in terms of euros per kg of fish, was higher with these diets, particularly with SB-IB, without fish meal, which also improves the sustainability of trout feeding.

Conclusion

The study investigated the impact of substituting fishmeal with organic byproducts, such as seabass, Iberian pig byproducts, and insects, in developing 100% organic diets for rainbow trout. The study findings indicate that the substitution of fishmeal influenced the growth performance of the fish, with the control diet yielding the best results followed by diets containing fishmeal of marine origin (SB). However, diets incorporating lower-cost animal byproducts demonstrated superior economic performance. Despite observed differences in growth, the high substitution of fishmeal did not significantly alter the composition of the intestinal microbiota, possibly due to the predominance of Firmicutes. Although histological changes were noted in fish-fed diets containing alternative protein sources, no significant differences were observed in the diet's intestinal microbiota composition. From an economic perspective, diets containing animal byproducts (SB-IB) exhibited the lowest economic conversion index and the highest economic profit index. These findings underscore the importance of considering both performance and economic factors when formulating organic diets for aquaculture, aiming for sustainable and economically viable production systems.

The economic advantages of SB-FM and SB-IB diets, particularly those without fish meal, contribute to the overall sustainability of trout feeding. The findings highlight the potential for cheaper organic diets, where the reduced cost of diets can compensate for lower growth. Therefore, these diets' profit index was higher, emphasizing the economic viability and sustainability of utilizing seabass and Iberian pig byproducts in rainbow trout aquaculture.

Acknowledgments

The first author was supported by a complete grant from the Ministry of Higher Education of the Arab Republic of Egypt, and the research project was developed with the collaboration of the Biodiversity Foundation (Spanish Ministry for Ecological Transition and the Demographic Challenge), through the Pleamar Program, co-financed by the European Maritime and Fisheries Fund (EMFF).

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General Discussion

General Discussion

The availability of feeds appropriate for organic production of carnivorous species presents a significant challenge to expanding organic aquaculture. This challenge is due to the constraints imposed by EU regulations and the limited availability of suitable raw materials for crafting well-balanced diets (Berge *et al*., 2015b; Mente *et al*., 2011a)).

The primary concern in formulating organic feeds is the quest for additional raw materials and alternatives to address the growing demand for protein sources, especially considering the fishmeal shortage (Enami, 2011; Hardy, 2010; T. G. Pereira & Oliva-Teles, 2003). However, in the context of organic aquaculture, the precise ratio for incorporating marine and plant-based proteins into the feed has not been clearly quantified (Mente *et al*., 2011a).

To eliminate captured fishmeal from aquaculture feeds and make them more sustainable, the incorporation of various organic animal protein ingredients has been studied in this thesis. The experimental design incorporated control diets and organic controls in evaluating various ingredients for aquaculture feed formulation. Following an assessment that validated the efficacy of the organic control, the decision was made to utilize the organic control. Different eco-products, selected in function of their availability, such as poultry meal, Iberian pig by-product, insect meal, and byproducts of trout and seabass filleting, were tested in various formulations for the feeding of gilthead seabream, seabass, and rainbow trout. Organic plant ingredients were also used, including wheat, pea flour, wheat gluten, and soybean meal. Growth curves were obtained for all the species studied, and the nutritional efficiency of the feeds was evaluated, including digestibility, digestive enzymes, body composition, microbiota composition, and intestinal histology.

Trout by-product meal has demonstrated efficacy as a dietary component, particularly when incorporated into gilthead seabream and sea bass formulations. Formulations encompassing trout meal, treated as a conventional fish ingredient, have exhibited favourable outcomes. Combinations incorporating trout as a constituent have consistently delivered positive results in various contexts.

In the initial experiment, sea bass by-product meal emerged as a highly efficacious ingredient for gilthead seabream. Although absent in the second experiment, the case of trout (fourth experiment) exhibited notable effectiveness. Notably, formulations featuring seabass byproducts meal proved to be the most successful. Conversely, poultry demonstrated suboptimal performance. Additionally, insect-based diets fared poorly across the second, third, and fourth experiments, consistently ranking among the least effective formulations.

The positive outcomes observed in fish-fed diets containing trout and seabass byproducts can be attributed to the inherent nutritional benefits of the diet. The fish protein derived from trout meal and seabass meal byproducts exhibits an amino acid profile closely aligned with the nutritional requirements of the fish, contributing to their improved growth. Beyond the nutritional aspect, the utilization of trout and seabass byproducts aligns with the principles of a circular economy, promoting resource efficiency and waste reduction (Cooney *et al.*, 2023). By incorporating these byproducts into other products or processes, the reliance on additional resources is minimized, and waste disposal is reduced, resulting in a favourable environmental impact. The establishment of a commercial organic supply chain for trout, seabass, and their byproducts holds the potential for enhancing the sustainability of production cycles and contributing to broader environmental conservation efforts. According to European regulations, by-products derived from organic aquaculture hold the second-highest priority, surpassed only by producing organic algae or other organic food items originating from aquaculture. Notably, chicken is assigned the fifth position in priority, sharing this ranking with insects. These guidelines are outlined in EU Regulation 2018/848.

Furthermore, the inclusion of organic alternative fish ingredients, such as rainbow trout, in the diets of fish represents a cost-effective and environmentally sustainable approach compared to traditional fishmeal or plant-based diets. This strategy diversifies feed sources and reduces dependency on conventional ingredients, thereby promoting a more sustainable aquaculture industry. While the supply of organic seabass and trout may not be substantial enough to warrant the establishment of dedicated organic meal factories, the creation of such facilities has the potential to enhance the profitability of organic production greatly. This initiative aligns to promote a more sustainable and environmentally conscious aquaculture sector. Realizing the full potential of by-products necessitates the implementation of separate facilities designed to process each ecological species. However, this undertaking poses a significant challenge to industrial development, primarily attributable to the limited quantity produced in organic farming. Despite these challenges, establishing specialized facilities remains crucial to advancing the organic aquaculture industry and fostering a more sustainable future.

Additionally, ensuring homogeneity in the nutritional composition of organic meals in each batch of production presents a significant challenge due to the inherent variability in organic feed ingredients. Factors such as variations in organic raw materials, sourcing practices, processing methods, and environmental conditions can contribute to fluctuations in nutritional content. While efforts can be made to standardize production processes and quality control measures, achieving absolute uniformity may be difficult in organic agriculture, where natural variability is more pronounced. Nevertheless, implementing stringent quality assurance protocols and closely monitoring production parameters can help minimize variations and enhance the consistency of nutritional composition across batches. Continued research and technological advancements in organic feed production may further improve the homogeneity of organic meals over time.

Iberian pork is more controversial. Diets containing Iberian pork, but also including another fish source, have worked well as a mixture. However, performance decreases when used alone or in combination with insect. When combined with some fish, as seen in the second experiment, the performance of Iberian pork improves. In the third experiment, Iberian pork has had poor performance, and the same goes for Iberian pork insect and the mixture. However, Iberian pork trout has performed well, as has the combination of trout and insect. Mixtures of seabass and Iberian pork, and the mixture of seabass, insect, and Iberian pork, have also performed well. The Iberian pig possesses a processing channel and quantities that would likely facilitate a more robust industrial development of these organic meals.

The underperformance of insect-based diets prompts consideration of potential contributing factors. Notably, the inherent heterogeneity of insects, influenced by species and various contextual factors, adds complexity to comparisons with other studies. Unlike homogeneous products, such as organic seabass, trout, and Iberian pork byproducts, insects exhibit variability as byproducts. The exceptional efficacy of seabass, trout, and Iberian pork byproducts, especially when amalgamated with certain ingredients, stands in contrast to the less favorable outcomes associated with insect-based diets. A plausible explanation for this disparity might be rooted in the insect's potential role as an antinutrient, suggesting a need for further investigation into the nuanced interactions within these dietary formulations.

Recognizing the potential of insect meals as a protein source, it is imperative to acknowledge their substantial chitin content. Chitin, a prominent component of insect exoskeletons, is notorious for its anti-nutritional characteristics, posing a challenge to efficient fish digestion (Barroso *et al*., 2014). Notably, chitin has been associated with hindered nutrient absorption, as exemplified by its adverse impact on tilapia's growth rate and feed conversion (Shiau *et al* 1999). The anti-nutritional effects of chitin are attributed to its resistance to enzymatic digestion in fish, leading to compromised protein breakdown and hindered energy release from the diet. It is crucial to underscore that the levels of anti-nutritional factors (ANFs) in insect meals can vary significantly based on insect species, rearing conditions, and processing methods.

Few studies have explored fishmeal substitution for organic diets, with some notable findings indicating potential alternatives. Lunger et al. (2006, 2007) demonstrated that up to 40% of fishmeal protein can be replaced by NuPro, an organically certified yeast-derived protein source, without compromising growth performance in juvenile cobia (Rachycentron canadum). Similarly, Di Marco et al. (2017) compared commercial and organic feeds for seabass and sea bream, incorporating organic soybean cake and wheat, and found comparable performance between the diets. This study revealed minimal environmental impact in organic farming, alongside improved growth and metabolic status in organic fish, despite a slightly higher incidence of fin splitting. Carminato et al. (2020) investigated European sea bass fed with organic and conventional diets, assessing growth, oxidative stress, and contaminant markers. Although both groups exhibited positive growth trends, conventional-fed fish showed greater productivity, while organic-fed fish displayed significantly higher expression of contaminant markers. Fillet analysis revealed differences in fatty acid composition, with organically fed fish exhibiting higher monounsaturated fatty acid content and lower polyunsaturated n-6 content.

In contrast, Estévez and Vasilaki (2023) explored the use of novel ingredients, including green pea protein and brown seaweed, as fish meal replacements in organic feed. Their trials on gilthead seabream showed no significant differences in growth, feed utilization, or fillet composition compared to the control commercial diet. Fish fed alternative ingredients exhibited higher muscle protein content and altered fatty acid composition, with increased levels of beneficial omega-3 and omega-6 fatty acids. Liver composition mirrored feed composition, suggesting the potential of these ingredients in organic feed formulations without adverse effects on fish growth or product quality. Despite these promising findings, the scarcity of studies on organic diet formulation may stem from the challenge of sourcing organic raw ingredients and the imperative to reduce reliance on fisheries-derived raw materials like fishmeal and oil to achieve sustainable aquaculture production.

Economic Productivity Assessment

In the study of availability and price, it was found that there is a limited supply of animal protein ingredients due to the lack of selective collection and treatment processes. This has posed challenges to the research development but also presents a potential future business opportunity. Another crucial aspect to consider is the economic profitability when incorporating different raw materials into fish feeds. Economic conversion index (ECI) and economic profit index (EPI) (Jauralde *et al.*, 2013; Martínez-Llorens *et al.*, 2017) have been calculated as follows:

$$\text{ECI (€/kg)} = \text{FCR} * \text{Price diet}$$

$$\text{EPI (€/kg)} = (\text{FW} * \text{SP}) - (\text{FCR} * \text{Weight Gain})$$

Where FCR is the feed conversion rate, FW is the final weight of the fish, and SP is the selling price of the fish in the market.

In ECI, it indicates the cost required to fatten one kilogram of fish, while EPI indicates the increase in added value produced by fattening one fish.

The prices of different feed studied in the case of fishmeal substitution are reduced except for the diets that have insects (Table 17). Previous experiments have shown that substituting alternative protein sources for fish meal reduces feed costs due to the high price of fish meal in the market (Martínez-Llorens *et al.*, 2012; Sánchez-Lozano *et al.*, 2009).

Regarding the economic indices obtained in this thesis (Table 17), substituting alternative protein sources is more profitable except in case of insect, as mentioned earlier, the value of poultry meal, trout meal, seabass meal, and Iberian pig meal is much lower than that of fish meal. While ECI depends heavily on the diet price, the economic profit index (EPI) seems more suitable for comparing economic profitability as it considers a greater number of parameters.

In Experiment 1, Trout (TRO) and seabass (SBS) exhibits a relatively low ECI (2.39 and 2.36 respectively), signifying efficient economic conversion, while the poultry (POU) diet displays a higher ECI (3.13). In Experiment 2, IBE and TRO maintains a low ECI (1.20 and 1.48), emphasizing its economic efficiency, whereas Insect (INS) demonstrates a higher ECI (4.12), implying elevated costs in economic conversion. Notably, IBE and TRO attains the highest Profit Index (8.80 and 8.52), indicating a favorable balance between costs and profits. Experiment 3 unveils that Iberian Pork-Trout (IB-TR) showcases a relatively low ECI (2.78) and a high Profit Index (8.22), indicating economic efficiency, while Iberian Pork-Insect (IB-IN) presents a higher ECI (6.51) and a lower Profit Index (4.49). Overall, combinations like IB-TR demonstrate a balance between economic conversion efficiency and profitability. In Experiment 4, SB-IB and SB-FM presents a positive Profit Index (5.23 and 4.85) and a moderately low ECI (2.77 and 3.15), whereas IN-IB has a low Profit Index (-1.96) and a high ECI (9.96), indicating potential economic challenges. General trends reveal consistent efficiency and high profit indices for trout and seabass, variability in Iberian Pork performance, and mixed results for Insect inclusion. ECI reflects cost-effectiveness, with lower values deemed desirable, while the profit index signifies economic profitability.

Table 17. The Economic Conversion Index and the profit Index in each of the tests carried out with the different species during the thesis.

Experiment 1	**CONT**	**ORG**	**TRO**	**SBS**	**MIX**	**POU**
Price of diet (€/kg)	1.50	1.76	1.03	1.04	1.07	1.07
Economic Conversion(€/kg)	2.60	3.35	2.39	2.36	2.56	3.13
Experiment 2	**CON**	**TRO**	**INS**	**IBE**		
Price of diet (€/kg)	1.39	0.94	3.20	0.97		
Economic Conversion(€/kg)	1.71	1.48	4.12	1.20		
Profit Index (€/kg)	8.29	8.52	5.88	8.80		
Experiment 3	**CON**	**IB**	**IB-IN**	**IB-TR**	**TR-IN**	**MIX**
Price of diet (€/kg)	1.57	1.23	2.47	1.18	2.42	2.03
Economic Conversion(€/kg)	3.17	2.98	6.51	2.78	5.76	4.30
Profit Index (€/kg)	7.83	8.02	4.49	8.22	5.24	6.70
Experiment 4	**Control**	**FM-IN**	**IN-IB**	**SB-FM**	**SB-IB**	**SB-IN-IB**
Price of diet (€/kg)	1.86	3.34	2.95	1.38	1.26	2.21
Economic Conversion(€/kg)	4.00	7.78	9.96	3.15	2.77	4.73

Profit Index (€/kg)	4.00	0.22	-1.96	4.85	5.23	3.27

When comparing EPI values with other studies with Mediterranean species, such as sea bream (M. D. Hernández *et al*., 2007; Lozano *et al*., 2007; Moutinho *et al*., 2017a), which presented values between 1.28 and 1.43 €/kg in the best feeds, respectively, or sea bass, with values of 1.8 €/kg (Adaklı & Taşbozan, 2015), or white seabream, with values of 1.5 €/kg (M. D. Hernández *et al*., 2007; Lozano *et al*., 2007; Moutinho *et al*., 2017a), it see that in all cases, they are much lower than the data obtained in this thesis. This indicates that the use of studied ingredients is a more economically profitable.

Organic poultry and insect meals did not yield satisfactory results. However, organic trout and seabass filleting byproducts, along with Iberian pig byproducts, showed excellent results in terms of growth and economic productivity. This opens significant prospects for the future of feeding various organic aquaculture species without using fishmeal, thereby making aquaculture more sustainable by reusing byproducts from organic livestock and aquaculture. Although a wealth of information has been obtained, further experimentation with new organic animal protein sources is necessary to optimize the feeding of different aquaculture species and achieve competitive and profitable organic aquaculture.

The inherent cost advantage of by-products, arising from their classification as such, positions them as economically favorable resources for various applications. Their lower prices make them financially viable and highly attractive for utilization in different industries. On the contrary, the current pricing of insect meal presents a considerable challenge, limiting its immediate suitability as a practical option. However, the higher cost associated with insect meal should be viewed through the lens of a forward-looking investment. While not presently a cost-effective choice, the potential of insect meal as a valuable resource in aquaculture becomes evident when considering ongoing advancements in production methods and scale. The key to unlocking its feasibility lies in the anticipation of a substantial reduction in prices, making insect meal an economically viable and practical solution for aquaculture applications in the future. As technological and agricultural developments progress, the trajectory of insect meal as a sustainable and efficient resource for aquaculture is likely to evolve, ushering in a new era of possibilities for the industry.

In relation to future perspectives**,** this thesis has demonstrated the feasibility of feeding and producing gilthead seabream, seabass, and rainbow trout using organic animal ingredients without fishmeal. This opens significant prospects for developing more economical and sustainable organic feeds by repurposing by-products from organic livestock and aquaculture.

It is now evident that this is the primary alternative for enhancing Spanish aquaculture production. However, it does entail addressing several challenges. The most prominent challenge is the availability of organic animal ingredients. Addressing this necessitates new initiatives to explore novel ingredients (feather hydrolysate, other organic meat meals, microbial protein, etc.) and processing methods (hydrolysis, fermentation, etc.), not only for animal products but also for organic plant-based ingredients (e.g., sunflower). Another challenge is increasing the demand for organic fish since it remains relatively unknown to eco-conscious consumers.

These studies highlight the potential of organic ingredients as substitutes for fishmeal in aquaculture diets, which could contribute to more sustainable and environmentally friendly practices. Further research and optimization of formulations, as well as exploring the inclusion of supplementary ingredients, are recommended to improve efficiency and fully assess the possibility of replacing 100% of fishmeal in these diets. This research contributes to the development of more sustainable and healthy aquaculture practices.

Conclusions

The conclusions from this doctoral thesis can be summarized as follows:

- ✓ Organic trout byproducts have consistently demonstrated efficacy as a dietary ingredient, especially in formulations for gilthead seabream and sea bass. Treating trout as a conventional fish ingredient, these formulations have yielded favourable outcomes.

- ✓ Organic seabass byproducts have emerged as a highly efficacious ingredient for gilthead seabream and rainbow trout.

- ✓ Organic poultry byproducts and organic insect meals demonstrated suboptimal performance.

- ✓ Organic Iberian pork byproducts is more controversial, with outcomes depending on combinations with other ingredients. Diets containing Iberian pig and another fish source have worked well, as have mixtures such as trout. However, performance decreases when Iberian pork is used alone or in combination with insects.

- ✓ The economic indices obtained in the study indicate that substituting fish meal protein by organic animal byproducts is generally more profitable, except in the case of insect-based diets. Prices of different feeds studied show a reduction, except for diets containing insects.

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