TESIS DOCTORAL

Valorización de insectos y su aplicación en dietas alternativas en acuicultura

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RESUMEN

Naciones Unidas prevé que la población mundial se incrementará hasta los 10.000 millones para el año 2050. Esta creciente población requiere que la industria alimentaria siga su ritmo de crecimiento. Dentro de la industria alimentaria, la que mayor incremento ha experimentado en la última década es la acuicultura. La producción acuícola ha dado un gran salto desde la década de los 90 hasta la actualidad, situándose a la par de la producción pesquera, la cual se ha estancado en el mismo periodo. La acuicultura ofrece un producto de alta calidad nutricional a un precio más accesible, pero no se encuentra exenta de problemáticas. Desde su auge, se han puesto bajo el foco los problemas de sostenibilidad que acompañan a la acuicultura, tales como el uso de la harina de pescado, el empleo de antibióticos, la destrucción de ecosistemas, la eutrofización, etc. En los últimos años, la búsqueda de alternativas sostenibles a la harina de pescado, debido principalmente al aumento de su precio, se ha multiplicado. La harina de insecto ofrece una alternativa proteica con una menor huella ecológica respecto a la proteína animal tradicional. No obstante, y aunque la producción de harina de insecto es más sostenible que la de harina de pescado, presenta limitantes, como carencias en algunos aminoácidos esenciales, niveles bajos o nulos de ácido eicosapentaenoico (20:5*n*3, EPA) y ácido docosahexaenoico (22:6*n*3, DHA), y digestibilidad proteica reducida debido a la presencia de quitina.

En esta tesis se ha enfocado la producción acuícola bajo un marco de sostenibilidad, por lo que se probó la adecuación de harina de *Tenebrio molitor* y *Hermetia illucens* en niveles de sustitución del 15 y 30 % en tres especies acuicultivadas (*Oncorhynchus mykiss*, *Sparus aurata* y *Tinca tinca*). Se evaluó el crecimiento, metabolismo proteico y calidad lipídica del filete. Se observó que, bajo estos niveles de sustitución, el metabolismo proteico y el crecimiento no se vieron especialmente afectados, mientras que los niveles de EPA y DHA disminuyeron al incluir las harinas de insecto. Junto con la evaluación inicial de la composición de los insectos, usados para la elaboración de las harinas de insecto, se evaluó la capacidad para mejorar el perfil lipídico de *H. illucens* y *T. molitor* mediante la ingesta de pescado de descarte rico en EPA y DHA. En este aspecto, se observó que *H. illucens* mostró una mejor capacidad de acumulación de estos ácidos grasos respecto a *T. molitor.* En relación con los porcentajes de sustitución, se observó que niveles superiores a los mencionados en dietas de *S. aurata* mostraron que los efectos negativos respecto al crecimiento se incrementaban, especialmente para las dietas compuestas por altos niveles de sustitución (50 %), tanto de harina de *H. illucens*

alimentada con pienso comercial como la alimentada con pescado de descarte. Por otra parte, y debido al impacto que tiene la población microbiana intestinal en el correcto desarrollo de los peces, se evaluó el efecto que puede ejercer la harina de insecto en el tracto intestinal de *S. aurata*. Así, se detectó que se modificó la composición del microbioma intestinal de *S. aurata*, estando ausentes algunas de las especies presentes en los individuos alimentados con dieta control: De las dietas empleadas, la que mayor similitud mostró, respecto a la dieta control, fue la compuesta por *H. illucens* alimentada con pescado de descarte. Estos resultados demuestran que la aplicación de niveles moderados de harina de insecto como alternativa proteica a la harina de pescado es posible; no obstante, hay que poner foco en la composición lipídica y elaboración de la harina de insecto para lograr mayores niveles de sustitución.

La quitina forma parte del exoesqueleto de los insectos y representa en ocasiones un limitante a la hora de incluir niveles superiores de sustitución de harina de pescado por harina de insecto. Una posible solución puede ser la separación de este componente menos digerible del resto del insecto. En este sentido, y al objeto de maximizar el aprovechamiento de las distintas fracciones del insecto, se evaluó la capacidad de un derivado de la quitina (quitosano), para tomar parte en la composición de una matriz de nanoencapsulación de polifenoles de té verde, como alternativa sostenible al uso de antibióticos en acuicultura. Se observó que la presencia de quitosano era el factor más relevante a la hora de mostrar actividad antibacteriana frente a cinco bacterias patogénas de peces. Por otro lado, la cobertura de quitosano les aportó estabilidad a las sustancias encapsuladas, al tiempo que permitió mantener una gran parte de su poder antioxidante.

ABSTRACT

The United Nations predicts that the world's population will increase to 10 billion by 2050. This growing population requires the food industry to keep pace with its growth. Within the food industry, the industry that has seen the greatest increase in the last decade is aquaculture. Aquaculture production has leapt from the 1990s to the present day, catching up with fish production, which has stagnated over the same period. Aquaculture offers a product of high nutritional quality at a more affordable price, although it has certain disadvantages. Since its rise in production, the sustainability problems that accompany aquaculture, such as the use of fishmeal, the use of antibiotics, destruction of ecosystems, eutrophication, etc., have come under the spotlight. In recent years, the search for sustainable alternatives to fishmeal, mainly due to its rising price, has multiplied. Insect meal offers a protein alternative with a smaller ecological footprint than traditional animal protein. However, although the production of insect meal is more sustainable than fishmeal, it has limitations, such as deficiencies in some essential amino acids, low or zero levels of eicosapentaenoic acid (20:5n3, EPA) and docosahexaenoic acid (22:6n3, DHA), and reduced protein digestibility due to the presence of chitin.

This thesis has focused on aquaculture production under a sustainability framework, therefore the suitability of *Tenebrio molitor* and *Hermetia illucens* meal was tested at substitution levels of 15 and 30 % in three species relevant in aquaculture (*Oncorhynchus mykiss*, *Sparus aurata* and *Tinca tinca*). Growth, protein metabolism and lipid quality of the fillet were evaluated. It was observed that, under these substitution levels, protein metabolism and growth were not particularly affected, while EPA and DHA levels decreased with the inclusion of the insect meals. Along with the initial evaluation of the insect composition used for insect meal production, the ability to improve the lipid profile of *H. illucens* and *T. molitor* by ingesting discarded fish rich in EPA and DHA was assessed. In this respect, it was observed that *H. illucens* showed a better capacity to accumulate these fatty acids than *T. molitor*. Higher levels of fishmeal substitution in *S. aurata* diets showed that the negative effects on growth increased, especially for diets composed of high levels of substitution (50%) of both *H. illucens* meal fed with commercial feed and *H. illucens* meal fed with discarded fish. On the other hand, the effect that insect meal can have on the intestinal microbiota of *S. aurata* was evaluated. Thus, it was detected that the composition of the intestinal microbiome of *S. aurata* was modified. Some of the microorganisms present in the fish fed with control diet being absent. Of the experimental diets used, the one that showed the greatest similarity with

respect to the control diet was that composed of *H. illucens* fed with discarded fish. These results demonstrate that the application of moderate levels of insect meal as a protein alternative to fish meal is possible. However, to achieve higher levels of substitution focus needs to be placed on the lipid composition and processing of the insect meal.

Chitin is part of the insect exoskeleton and could represent a constraint when including higher levels of fishmeal substitution for insect meal. A possible solution may be to extract this less digestible component from the rest of the insect. In this sense, and in order to maximize the utilization of the different fractions of the insect, the capacity of a chitin derivative (chitosan) as part of a green tea polyphenol nanoencapsulation matrix was evaluated as a sustainable alternative to the use of antibiotics in aquaculture. The presence of chitosan was found to be the most relevant factor in showing antibacterial activity against five fish pathogenic bacteria. On the other hand, the chitosan coating provided stability to the encapsulated substances, while allowing them to maintain a large part of their antioxidant power.

INTRODUCCIÓN

1.1. Estado de la acuicultura

1.1.1. Acuicultura global

La población humana se espera que llegue hasta los 10.000 millones para el año 2050, según datos de la Organización de las Naciones Unidas (United Nations, 2019). El aumento de la población trae consigo un aumento de la demanda de proteína. La proteína animal representa, de media, el 39 % del total de proteína consumida en el mundo en el año 2020. Este consumo ha aumentado en un 63 % desde el año 1960 (FAOSTAT, 2023). A nivel mundial, el consumo medio de pescado, moluscos y crustáceos por año año ha aumentado desde los 8,9 kg por persona en 1960 hasta los 20,2 kg por persona en 2020; esto supone actualmente un 18 % del total de proteína animal consumida en el mundo (FAOSTAT, 2023). En este contexto de aumento en la demanda de proteína, la acuicultura ha crecido un 300 % desde la década de los 90 hasta el año 2020, pasando de una producción de 21,8 hasta 87,5 millones de toneladas (FAO, 2022). Ese consumo no ha aumentado del mismo modo para toda la población. En el estudio llevado a cabo por Andreoli et al. (2021), se destaca que el consumo de productos derivados de animales aumenta conforme aumenta la renta de un país, si bien, al alcanzarse rentas muy altas, este consumo disminuye. De los datos disponibles podemos extraer que los países con renta baja han disminuido su consumo medio por persona de proteína animal en un 5 %, los países con rentas bajas-medias han aumentado su consumo de proteína animal en un 160 %, mientras que los países con rentas media-alta y alta han aumentado su consumo en un 185 y un 44 %, respectivamente (FAOSTAT, 2023). Teniendo en cuenta que los países de renta media-baja (países en desarrollo) son los más poblados, la FAO estima que las necesidades de alimentos se incrementarán de manera exponencial, y no proporcionalmente, respecto al aumento de la población, por lo que se espera un aumento del consumo de pescado. Este aumento que se prevé no es soportable únicamente por la pesca, como veremos más adelante, y ha de ser cubierto por la acuicultura que, además, deberá hacerlo de forma sostenible.

Según la FAO (FAO, 2022):

“La acuicultura es la cría de organismos acuáticos, incluidos peces, moluscos, crustáceos y plantas acuáticas. La acuicultura implica algún tipo de intervención en el proceso de cría para mejorar la producción… La cría también implica la propiedad individual o corporativa de la población que se cultiva. A efectos estadísticos, los organismos acuáticos recolectados por una persona física o jurídica que ha sido su propietaria durante

todo el periodo de cría pertenecen a la acuicultura, mientras que los organismos acuáticos que pueden ser explotados por el público como recursos de propiedad común, con o sin las licencias pertinentes, pertenecen a la pesca."

Actualmente la acuicultura produce el 50 % del total de la producción de pescado, moluscos y crustáceos en el mundo. Si también tenemos en consideración la producción de algas, la acuicultura aumenta al 57 % del total de la biomasa obtenida en 2020 (FAO, 2022). Esto sitúa a la acuicultura como una de las industrias alimentarias que más ha crecido en las últimas décadas (Garlock et al., 2019).

Por otro lado, según datos de la FAO (FAOSTAT, 2023), la media del crecimiento anual de la industria pesquera entre el año 1996 y el 2020 es 0,02 %, lo que significa que la industria pesquera no crece, manteniendo su producción prácticamente constante (**Figura 1**). La industria pesquera está dominada por China, con un 16 % del total de la producción pesquera global, si bien su media de crecimiento anual (1995-2020) es menor a la de los países que le siguen en producción, Indonesia y Perú (FAOSTAT, 2023).

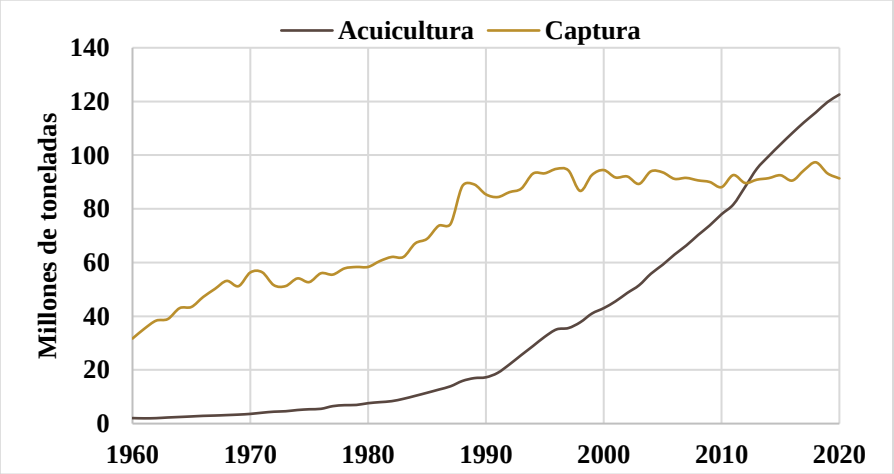


Figura 1. *Evolución en el periodo 1961-2020 de la producción pesquera y acuícola (incluyendo algas). Datos obtenidos de FAO (FAOSTAT, 2023).*

Aunque la producción en acuicultura (excluyendo algas) supone cerca del 50 % del total, el aporte no es equitativo globalmente. Por continentes, Asia ocupa el primer puesto, con una producción acuícola del 91,6 %, seguida de América del Norte y Sur (3,59 %), Europa (2,69 %), África (1,92 %) y Oceanía (0,19 %) (FAO, 2022).

En la **Figura 2** podemos ver desglosada la media de la producción acuícola, incluyendo algas, en distintos periodos desde el 1996 hasta el 2020. Se ha representado la producción global, la de China y la producción global sin China. La tendencia muestra cómo, aun siendo el mayor productor mundial, la distancia que separa a China de la producción global excluyéndola se ha reducido desde un 211 % la década de los 90, hasta el 134 % en 2020. Esto indica un crecimiento de la acuicultura global más allá de China.

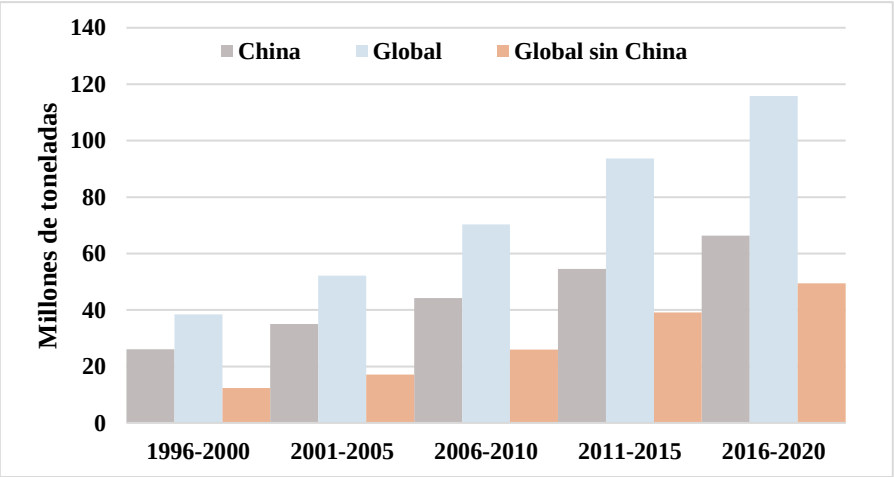


Figura 2. *Media de la producción acuícola (en millones de toneladas) en distintos periodos. Datos obtenidos de FAO (FAOSTAT, 2022).*

El crecimiento anual para el mundo sin la inclusión de China muestra un claro aumento de la media de producción anual desde el año 1996 hasta el 2005, disminuyendo levemente desde ese punto hasta el año 2015 (FAOSTAT, 2023). Las razones de esta disminución general para el periodo 2016-2020, en los tres casos analizados, se asocian principalmente a la pandemia global provocada por el virus COVID-19, afectando principalmente a la cadena de suministro en la acuicultura y a la distribución del producto, reduciendo de este modo el crecimiento a nivel global (Ahmed and Azra, 2022; Monirul Alam et al., 2022).

1.1.2. Acuicultura en la Unión Europea

La Unión europea solo representa el 0,89 % del total de la producción mundial en acuicultura y un 4,4 % del total mundial de la industria pesquera. El crecimiento medio anual del 0,09 % (1995-2020) no arroja mejores resultados, mientras que el crecimiento anual del total de capturas para el mismo periodo disminuyó hasta un 2,2 % de media. En

la **Figura 3** se puede observar la producción anual del sector acuícola y pesquero en la Unión europea desde el año 1960 al 2020.

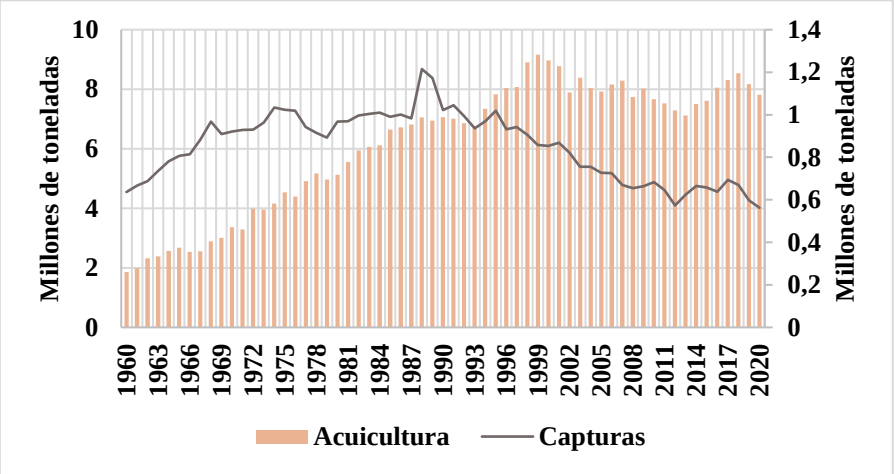


Figura 3. *Producción en millones de toneladas anual procedentes de la pesca (eje izquierdo) y de la acuicultura (eje derecho). Datos obtenidos de FAO (FAOSTAT, 2023).*

Durante la pandemia COVID-19 (periodo 2019-2020), se produjo una reducción del crecimiento de un 4,33 % y un 8,33 % de la producción acuícola y la pesca, respectivamente. Tras la pandemia, en febrero del año 2022, estalló el conflicto ruso-ucraniano, suponiendo un aumento en el precio medio de la energía, recomendaciones de no faenar en zonas cercanas al conflicto como el mar Negro, y una disrupción de las cadenas de suministro con Rusia (European Parliamentary Research Service, 2022).

Estas dos situaciones imprevisibles han truncado las proyecciones que la Unión europea tenía sobre su industria acuícola y pesquera. Estas proyecciones han ido sujetas a programas de subvenciones realizadas por la Unión (Guillen et al., 2019). La Unión Europea ha lanzado a lo largo de varias décadas programas de subvenciones destinados a la mejora del sector acuícola y pesquero. El primer gran programa de subvenciones (Financial Instrument for Fisheries Guidance, FIFG) fue puesto en marcha en el periodo 1994-2006, y se lograron resultados positivos en términos de modernización del sector, y mejora de la calidad higiénico-sanitaria, así como un aumento de beneficios en las empresas que recibieron fondos. El siguiente programa de subvenciones (European Fisheries Fund) se dio entre el periodo 2007-2014. Del total aprobado, se destinaron 600 millones de € a la industria acuícola, principalmente para aumentar la producción,

mediante la modernización de las instalaciones, la evaluación del impacto ambiental y el bienestar animal. En el periodo 2014-2020 se estableció otro programa de subvenciones (European Maritime and Fisheries Fund, EMFF), en el cual la cantidad asignada al sector acuícola fue de 1.725 millones, destinados principalmente al establecimiento y desarrollo de un sector acuícola sostenible.

El último programa de subvenciones (European Maritime, Fisheries and Aquaculture Fund, EMFAF) empezó en el año 2021 y contempla financiación hasta el año 2027. Como objetivo general, el programa planea el desarrollo sostenible de la acuicultura y la pesca, centrándose en actividades con emisiones bajas de carbono, protección de la biodiversidad en los ecosistemas, producción de productos de alta calidad y saludables, así como mejora técnica y de las condiciones laborales del sector (The European Maritime Fisheries and Aquaculture Fund, 2021a). Del total de fondos contemplados en el proyecto (5.311 millones de €), España recibirá más de 1.120 millones de €, siendo nuevamente el miembro de la Unión con una mayor financiación. Además de los fondos proporcionados por el EMFAF, España contribuirá con más de 453 millones de €. Del total, España invertirá en el sector acuícola aproximadamente 500 millones de € (The European Maritime Fisheries and Aquaculture Fund, 2021b). Como se aprecia en los diferentes programas. se ha pasado de unos objetivos de mejora de las condiciones y aumento de la producción a unos objetivos más relacionados con la sostenibilidad y bienestar.

1.1.3. Especies empleadas

Las especies empleadas para la realización de la presente tesis fueron dos especies de agua dulce: trucha arcoíris (*Oncorhynchus mykiss* Walbaum, 1792) y tenca (*Tinca tinca* Linnaeus, 1758), mientras que la dorada (*Sparus aurata* Linnaeus, 1758) fue la especie seleccionada proveniente de hábitat marino. Tanto la trucha arcoíris como la dorada son especies carnívoras, mientras que la tenca es una especie omnívora.

Respecto a su producción acuícola en la Unión Europea, la trucha se sitúa como la principal especie de pez producida en 2020, seguida por la dorada, con un total producido de más de 183.000 y 93.000 toneladas, respectivamente (FAOSTAT, 2023). Estos mismos datos sitúan a España como el cuarto productor de la Unión Europea de dorada y quinto productor de trucha arcoíris. La producción de tenca se sitúa en una escala menor en la Unión Europea, produciéndose en 2020 un total de 729 toneladas (FAOSTAT, 2023), siendo España el octavo productor a nivel de la Unión Europea.

1.2. Sostenibilidad en la acuicultura

Tal y como hemos comentado previamente, se está produciendo un aumento en el consumo de proteína animal a nivel global. Esto explica el aumento en la demanda de proteína animal. Según el informe de las Naciones Unidas (World Bank, 2013), se prevé un aumento de aproximadamente el 60 % de la producción en acuicultura, mientras que el consumo proyectado aumentará un 27 %. Este aumento se debe a que la industria acuícola provee de un producto rico en nutrientes a un precio asequible, aumentando la accesibilidad de estos productos a un mayor público. Con relación a las industrias ganaderas, la acuicultura presenta niveles similares o inferiores de impacto ambiental y eficiencia (Edwards, 2015; Little et al., 2016; Fry et al., 2018; Poore y Nemecek, 2018).

Aunque el impacto ambiental sea similar o menor que el de la ganadería terrestre, la acuicultura no es actualmente sostenible, y su práctica sigue suponiendo un alto impacto ambiental, principalmente en los ecosistemas donde se práctica. Los programas destinados a aumentar la producción acuícola van acompañados de medidas para mejorar la sostenibilidad de esta. Entre los principales impactos ambientales que supone la acuicultura, podemos destacar la eutrofización, el uso de harina y aceite de pescado, la utilización de antibióticos, la reducción de la biodiversidad y la destrucción de ecosistemas (Diana, 2009; Martinez-Porchas y Martinez-Cordova, 2012; Thurlow et al., 2019; Carballeira Braña et al., 2021).

1.2.1. Harina de pescado, un recurso finito

La harina de pescado es una fuente rica en proteínas de alto valor biológico, con un balance adecuado de aminoácidos, energía, minerales y vitaminas. Además, es una materia con alta digestibilidad proteica y alta palatabilidad, y con bajos niveles de componentes antinutricionales. Se produce a partir de pescado y/o subproductos de pescado no destinado al consumo humano, por no tener valor comercial o no tener el tamaño adecuado (Cho y Kim, 2011). Las principales fuentes usadas para la fabricación de la harina de pescado son peces pelágicos. El destino principal de la harina de pescado es la acuicultura (86 %), la ganadería (9 %) y la alimentación de mascotas (4 %) (FAO, 2022). En la **Figura 4** se aprecia el aumento del precio medio anual de la harina de pescado. La tendencia alcista desde el año 1999 se puede relacionar directamente con el aumento de la demanda, originada por el auge del sector acuícola, junto con las medidas restrictivas sobre las especies usadas para la fabricación de harina de pescado.

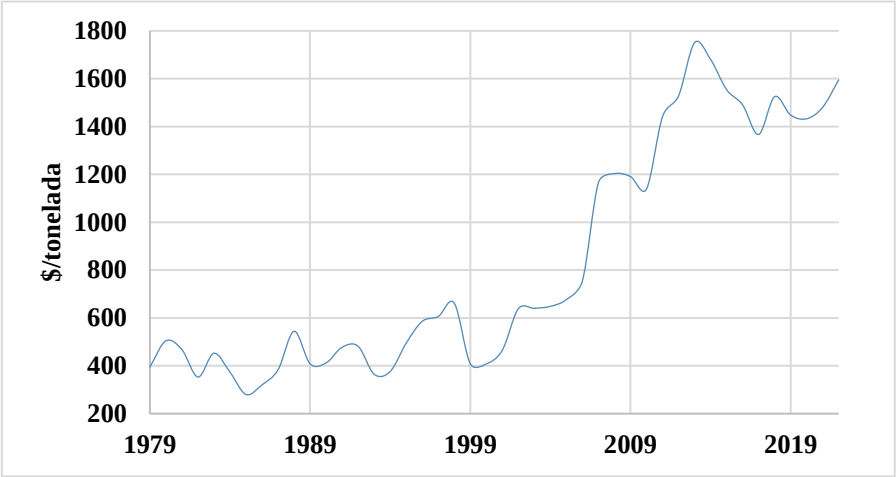


Figura 4. *Evolución del precio medio de la harina de pescado. Datos FAO (FAOSTAT, 2022).*

La dependencia de harina de pescado en la formulación de piensos para peces supone uno de los principales aspectos limitantes para el crecimiento sostenible de la acuicultura (Martinez-Porchas y Martinez-Cordova, 2011; Salin et al., 2018; Carballeira-Braña et al., 2021). El aumento de su demanda ha provocado una mayor captura de las especies destinadas a su fabricación, llevando a la sobreexplotación y reducción de poblaciones (Jannathulla et al., 2019; Canales et al., 2020; Shannon y Waller, 2021). Estas poblaciones juegan un papel importante en los ecosistemas que habitan, ya que sirven de alimento a animales situados en niveles superiores de la cadena trófica (Alder et al., 2008; Pikitch et al., 2014).

La cantidad de harina de pescado usada en las dietas formuladas en acuicultura varía según la especie y el sistema de cría (5-80 %) (Tacon y Metian, 2008). Aunque la demanda total de harina de pescado para la acuicultura ha crecido, el porcentaje de inclusión de harina de pescado en los piensos para peces ha disminuido, debido principalmente a dos estrategias: el ajuste de la relación proteína/energía, que procura una máxima utilización de aminoácidos con fines de crecimiento y no como fuente de energía, y la utilización de fuentes alternativas de proteína a la harina de pescado. De hecho, en su revisión, Tacon y Metian (2008) concluyen que la harina de pescado no es un ingrediente imprescindible en la alimentación de peces omnívoros, y debido a los altos precios y los impactos de la sobrepesca, recomiendan el uso fuentes alternativas de proteína. Destacan que la harina de pescado podría utilizarse como una harina de alta calidad destinada para piensos de inicio, acabado, y para reproductores.

La búsqueda de alternativas proteicas a la harina de pescado en acuicultura ha dado lugar a una de las corrientes de innovación más importantes en el sector. Esta búsqueda debe hacerse teniendo en cuenta, principalmente, los niveles de proteína, el perfil aminoacídico y la presencia de compuestos antinutricionales. Junto a las características anteriores, se considera que las fuentes alternativas de proteína deben de ser sostenibles (Mitra, 2021).

Actualmente se utilizan varias harinas con alto contenido proteico como alternativa a la harina de pescado. En este ámbito destaca la proteína de origen vegetal, especialmente la soja, siendo una de las fuentes proteicas más usadas en acuicultura, junto a la harina de pescado. La producción de harina de soja es más económica que la harina de pescado. Sin embargo, presenta puntos negativos, como la presencia de compuestos antinutricionales, que disminuyen la digestibilidad de los nutrientes, un contenido en ácidos grasos omega-3 poliinsaturados de cadena larga (*n*-3 LCPUFA) nulo, y altos niveles de carbohidratos solubles y estructurales. Además, la producción de soja no está exenta de problemas de sostenibilidad (Chou et al., 2004; Sánchez-Muros et al., 2014; Gu et al., 2016). Diversos estudios han revelado que la inclusión de harina de soja a distintos niveles afecta al crecimiento de los animales, así como a otros parámetros digestivos. Aunque hay que destacar que está disminución afecta de forma diferente a cada especie, debido a las diferencias en los hábitos alimentarios que presentan cada una de las especies, y el tratamiento y calidad de la soja utilizada (Refstie et al., 1997; Venou et al., 2006; Martínez-Llorens et al., 2007; Tomás-Vidal et al., 2010; García et al., 2013).

Dentro de las harinas de origen animal, encontramos los subproductos de animales, principalmente de la industria ganadera y alimentaría. Las ventajas que supone esta biomasa respecto a la harina de origen vegetal residen en su alto valor nutritivo, ya que poseen un nivel alto de proteína, buen perfil aminoacídico, ausencia de componentes antinutricionales y alta digestibilidad (Li y Wu, 2020; Woodgate et al., 2022). Esta mejor calidad proteica permite una mayor sustitución por harina de pescado en dietas de acuicultura. La aplicación de estas harinas ricas en proteína en acuicultura es extensa, siendo estudiados en diversas especies de la industria acuícola (Woodgate et al., 2022).

1.2.2. Nuevas Fuentes Alternativas

1.2.2.1. *Macroalgas y microalgas*

Las algas son un grupo de organismos marinos y de agua dulce con capacidad de realizar fotosíntesis y formar carbohidratos a través de CO_2. Estas carecen de tejidos presentes en

el reino vegetal y su tamaño puede variar desde una micra hasta varios metros. Las macroalgas se dividen en tres grupos: algas verdes (Chlorophyta), algas rojas (Rhodophyta) y algas marrones (Phaeophyta). Su uso se ha extendido en los últimos años, debido a su estudio en la producción de bio-diésel, su aplicación en la industria alimentaria, y la obtención de metabolitos secundarios (Leandro et al., 2019). La composición de las macroalgas varía según la especie; las algas rojas presentan un mayor contenido en proteína (10-47 %), las algas marrones contienen, por lo general, niveles más bajos (3-15 %), mientras que los niveles de proteína de las algas verdes se sitúan en una posición intermedia entre las dos anteriores (9-26 %) (Sudhakar et al., 2018). Si nos centramos en el perfil de aminoácidos esenciales, destacan las algas verdes y rojas, con un perfil aminoacídico más equilibrado (Martinez-Henandez et al., 2017; Viera et al., 2018). Su aplicación en acuicultura está limitada, debido principalmente a los costes de producción, a los altos niveles de carbohidratos, a la presencia de compuestos antinutricionales, y a la elevada concentración de metales pesados (Garcia-Vaquero y Hayes, 2016).

Las microalgas, por su parte, se pueden clasificar en algas verdes (Chlorophyta), algas rojas (Rhodophyta) y el resto de las microalgas (Chromophyta). Estos tres grupos agrupan las microalgas eucariotas, mientras que existe un grupo de microalgas procariotas denominado algas verdeazuladas (Cyanobacteria). Su uso ha ganado relevancia, al igual que para las macroalgas, en sectores como la producción de biodiésel, la industria alimentaria o la obtención de metabolitos secundarios para su uso en la industria farmacéutica (Camacho et al., 2019). Las microalgas son organismos ricos en proteína, vitaminas, metabolitos secundarios con actividad biológica, y a diferencia de las fuentes vegetales, *n*-3 LCPUFA, especialmente ácido eicosapentaenoico (20:5*n*3, EPA) y, en menor medida, ácido docosahexaenoico (22:6*n*3, DHA), y cierto tipo de aminoácidos esenciales (Shah et al., 2017; Naggapan et al., 2021). Las microalgas se han usado en acuicultura como medio de alimentación para rotíferos, copépodos y *Artemia* spp. Estos sirven de alimento en estados iniciales de peces, moluscos y crustáceos. Respecto a su contenido en proteína, lípidos y carbohidratos, estos varían según la especie de microalga. Para la proteína obtenemos un rango de 18-65 %, en lípidos podemos esperar una variación entre 5-48 %, y en carbohidratos la tendencia es la misma, con un rango que va desde un 5 hasta un 46 % (Brown, 1991; Tibbetts et al., 2015; Paggi-Matos et al., 2016; Niccolai et al., 2019). Esta variación en la composición se da según la especie, además de

darse también entre organismos de la misma especie, debido principalmente a las condiciones de cultivo. Es importante remarcar que esta variación en los niveles de proteína puede ser debida a una sobreestimación de esta. La mayoría de los análisis obtienen experimentalmente los niveles de nitrógeno, y la cantidad de proteína se estima a partir del contenido de nitrógeno, lo que puede llevar a considerar como proteína compuestos que no son, tal como aminas, ácidos nucleicos, glucosaminas y componentes nitrogenados de la pared celular (Becker, 2007). En los últimos años se han usado diversas especies de microalgas en alimentación en acuicultura, tales como *Spirulina* spp., *Scendesmus* spp., *Dunaliella* spp., *Chlorella* spp., etc. En su estudio, Vizcaino et al. (2014), probaron cuatro niveles de inclusión de *Scenedesmus almeriensis* (12-39 %) en dietas de dorada, concluyendo tras el experimento que no se observaron diferencias de crecimiento, ni en la utilización proteica entre las dietas con microalga respecto a la dieta control. Sáez et al. (2022) llevaron a cabo un experimento con *Nannochloropsis gaditana* a dos niveles de sustitución (5-10 %) en dietas de dorada, y no observaron diferencias en crecimiento, utilización proteica y composición en los individuos alimentados con dietas con microalga respecto al pienso control. Adicionalmente, obtuvieron un aumento en los niveles de EPA y DHA en aquellos animales alimentados con las dietas experimentales. En otro estudio, realizado en este caso en trucha arcoíris, la inclusión de *Scenedesmus almeriensis* hasta un 10 % generó efectos negativos en los índices de crecimiento para todos los niveles de inclusión probados, y efectos sobre los índices de utilización proteica en los niveles máximos de inclusión (Tomás-Almenar et al., 2018).

En definitiva, las algas, y las microalgas en particular, pueden ser una fuente interesante de EPA y DHA, y usarse como suplemento a niveles bajos de inclusión. La presencia de compuestos antinutricionales, el alto coste de procesado de la biomasa y pared celular rígida, que dificultan la biodisponibilidad de los nutrientes durante la digestión, hacen que su aplicación en acuicultura sea aún limitada (Camacho et al., 2019; Han et al., 2019).

1.2.2.2. Levaduras

Las levaduras son hongos unicelulares que se reproducen asexual y sexualmente. Actualmente se conocen más de 500 especies de levaduras, pertenecientes a unos 50 géneros, siendo *Saccharomyces cerevisiae* la especie más estudiada. El ser humano ha aprovechado durante miles de años la actividad fermentativa de las levaduras para elaborar cerveza, vino y pan. Las principales ventajas que presentan estos organismos unicelulares es su capacidad de transformar materia rica en fibra, de difícil digestión, en

nutrientes más fáciles de asimilar y aprovechar (Agboola et al., 2020). Para llevar a cabo esta transformación de material lignocelulósico, es necesario aplicar un pretratamiento con el fin de hidrolizar mediante medios físicos y/o químicos estos azúcares estructurales hasta azúcares fermentables (Agboola et al., 2020). Este proceso permite convertir subproductos de agricultura en biomasa con alto valor nutritivo. Aunque *S. cerevisiae* es la especie más usada, en los últimos años se han estudiado otras especies para su aplicación como biomasa alimentaria (Hatlen et al., 2012; Øverland et al., 2013; Chen et al., 2019). La composición de estas levaduras varía según la especie, aunque también varía dentro de una misma especie según el sustrato utilizado para su crecimiento. Los niveles de proteína varían desde un 17 hasta un 56 % (Øverland et al., 2013; Chen et al., 2019; Agboola et al., 2020), aunque está proteína suele estar sobreestimada, por el mismo motivo mencionado en las microalgas. En este caso, el porcentaje de nitrógeno asociado a ácidos nucleicos supone entre 10 y 25 % (Agboola et al. 2020). El perfil aminoacídico es similar al de la harina de pescado, aunque es común, entre las especies estudiadas, la carencia de metionina (Øverland y Skrede, 2017). Respecto a los lípidos, normalmente contienen valores bajos, que van desde 0,3 hasta 9 %, y con respecto a su composición, a diferencia de la harina de pescado, no aparecen *n*-3 LCPUFA, EPA y DHA (Brown et al., 1996; Halasz y Lasztity, 2017). Los carbohidratos totales no suelen superar el 30 % (Halasz y Lasztity, 2017).

Las levaduras se han suministrado en la alimentación animal desde hace más de 70 años, debido a sus propiedades inmunoestimulantes y probióticas. En acuicultura, su uso ha comenzado a tomar relevancia en los últimos años, debido a que estos organismos presentan altos niveles de proteína, y a que actúan favoreciendo el crecimiento, debido a la presencia de péptidos, nucleótidos y polisacáridos estructurales de la pared celular, como oligosacáridos de manano (Gatesoupe, 2007; Murthy et al., 2009; Manoppo et al., 2011). La inclusión (30 %) de biomasa de *S. cerevisiae,* como subproducto de fermentación, en dietas de dorada dio lugar a un aumento en los índices de crecimiento y en la utilización proteica respecto a los animales alimentados con dieta control (Estévez et al., 2022). En experimentos realizados en trucha arcoíris, el uso de levaduras a dos niveles de inclusión (10 y 20 %) supuso diferencias positivas en el crecimiento respecto a la dieta control, y también se observó aumento de los niveles de lípidos y proteína en el músculo de los animales, para ambos niveles de inclusión respecto al control. En lo concerniente al perfil de ácidos grasos, la inclusión de *S. cerevisiae* dio lugar a una

disminución de EPA y DHA en el músculo del animal, más pronunciada al aumentar el nivel de inclusión (Estévez et al., 2022). En otro estudio con niveles de inclusión de 11-32 % de *S. cerevisiae* y 12-35 % de una mezcla de *S. cerevisiae* + *Wickerhamomyces anomalus*, los autores concluyeron que niveles de inclusión de 21 y 24 %, para ambas combinaciones de levaduras, pueden usarse en dietas de trucha arcoíris sin afectar al crecimiento y la fisiología del intestino (Vidakovic et al., 2020).

En definitiva, la inclusión de levaduras en la sustitución parcial de la harina de pescado puede ser una estrategia recomendable, aunque, al igual que ocurría con las microalgas, varios factores limitan su aplicación. Por un lado, la presencia de compuestos antinutricionales, y de una pared celular rígida dificultan el aprovechamiento nutricional y condiciona una baja digestibilidad (Øverland y Skrede, 2017; Agboola et al., 2020; Sharif et al., 2021).

1.2.2.3. Bacterias

Las bacterias son organismos unicelulares procariotas, y constituyen el reino más numeroso en la tierra, tanto en número de especies como en masa total. El interés que reciben las bacterias para su uso en la alimentación es debido a su alto contenido en proteína, con un balance adecuado de aminoácidos esenciales, aunque con niveles de lisina inferiores, respecto a otras fuentes proteicas, bajos niveles de lípidos y crecimiento rápido (Bandara, 2018). Respecto a su cultivo y producción, cabe destacar que, al igual que ocurre en las levaduras, las bacterias pueden revalorizar subproductos provenientes de otras industrias alimentarias, siendo su eficiencia de conversión más alta que para las levaduras (Jones et al., 2020). Otro aspecto por destacar es la capacidad de utilizar como sustrato nutricional subproductos de la producción de hidrocarburos (Woolley et al., 2023). Los niveles de proteína de estas bacterias pueden llegar hasta el 80 % del total de la biomasa seca, mientras que los niveles de lípidos se mantienen en un rango de 1-3 % (Ritala et al., 2017). Las especies más relevantes son aquellas con capacidad de utilizar hidrocarburos como sustrato. La inclusión de biomasa de *Methylococcus capsulatus* en dos niveles (5 y 10 %) en dietas de dorada no supuso cambios en el crecimiento, ni en parámetros de utilización de la dieta, así como tampoco se apreciaron modificaciones en la composición proximal de los animales. Los niveles de EPA y DHA aumentaron en el músculo de los animales en dietas con *M. capsulatus* al 5 % respecto a la dieta control (Carvalho et al., 2023). En otro estudio, esta vez llevado a cabo con *Corynebacterium glutamicum* a tres niveles de inclusión (10-20 %), como sustituto de proteína vegetal, no

se observaron diferencias en parámetros de crecimiento, ni en utilización proteica de la dieta (Marchi et al., 2023). Investigaciones en trucha arcoíris con proteína bacteriana comercial con cuatro niveles de inclusión, donde el nivel de inclusión máximo supuso la sustitución del 100 % de la harina de pescado, supuso un aumento en parámetros de crecimiento y utilización de la dieta, para los dos niveles más bajos de sustitución por harina de pescado (25 y 50 %). La dieta donde se sustituyó el 100 % de harina de pescado por proteína bacteriana, originó una drástica reducción y empeoramiento de todos los parámetros estudiados. Los mismos resultados se observaron para la composición proximal y perfil de ácidos grasos, destacando la reducción en el contenido de proteína, lípidos totales, EPA y DHA (Zamani et al., 2020). En su estudio, Kiessling y Askbrandt (1993) evaluaron la inclusión de dos fuentes de proteína bacteriana (*Bacterium glutamaticum* y *Brevibacterium lactofermentum*) en dietas de trucha arcoíris en tres niveles de inclusión (4-16 %). Los datos de crecimiento y utilización de la dieta mostraron una disminución, para todas las dietas compuestas con diferentes niveles de inclusión de las dos fuentes proteicas bacterianas, siendo esta disminución más pronunciada en las dietas con *B. Glutamaticum*.

Al igual que ocurre con las microalgas y las levaduras, esta fuente proteica comparte los mismos problemas que las dos anteriores: la presencia de pared celular y de compuestos antinutricionales, y un alto contenido de ácidos nucleicos, que suponen la necesidad, en la mayoría de los casos, de un paso de procesado previo a su utilización (Ritala et al., 2017; Jones et al., 2020).

1.2.2.4. Insectos

Los insectos pertenecen al reino animal, filo Arthropoda y subfilo Hexapoda. Los organismos pertenecientes al filo Arthropoda son los organismos más numerosos en cuanto a especies descritas en el reino animal (**Figura 5**); cuentan con más de 1.200.000 especies, que representa alrededor del 75 % del total de especies descritas en este reino. El filo Arthropoda se divide en cuatro subfilos denominados Hexapoda, Chelicerata, Crustacea y Myriapoda, con 1.029.741, 113.894, 66.914 y 11.885 especies respectivamente. El subfilo Hexapoda se divide a su vez en cuatro clases donde la clase Insecta es la más numerosa. La clase Insecta se divide en diferentes ordenes donde destacan Coleoptera (387.100 especies), Lepidoptera (157.424 especies), Diptera (159.294 especies), Hymenoptera (116.861 especies), Hemiptera (103.590 especies) y Orthoptera (24.276 especies) (Zhang, 2011).

La entomofagia (consumo de insectos por parte de los seres humanos) es una práctica que ha acompañado al ser humano a lo largo de toda su historia, y sigue siendo relevante en diversas regiones del mundo, principalmente en Asia, América del Sur y Centro América, y África. Del total de especies descritas en la clase Insecta, más de 2000 especies son consumidas habitualmente en el mundo, de las cuales se pueden destacar los escarabajos (31 %), Lepidoptera (17 %), hormigas, abejas y avispas (15 %) y saltamontes (13 %) (van Huis, 2016). Aunque los insectos han sido participes en la alimentación humana durante milenios, su aplicación en el sector ganadero y acuícola ha tomado relevancia en los últimos años.

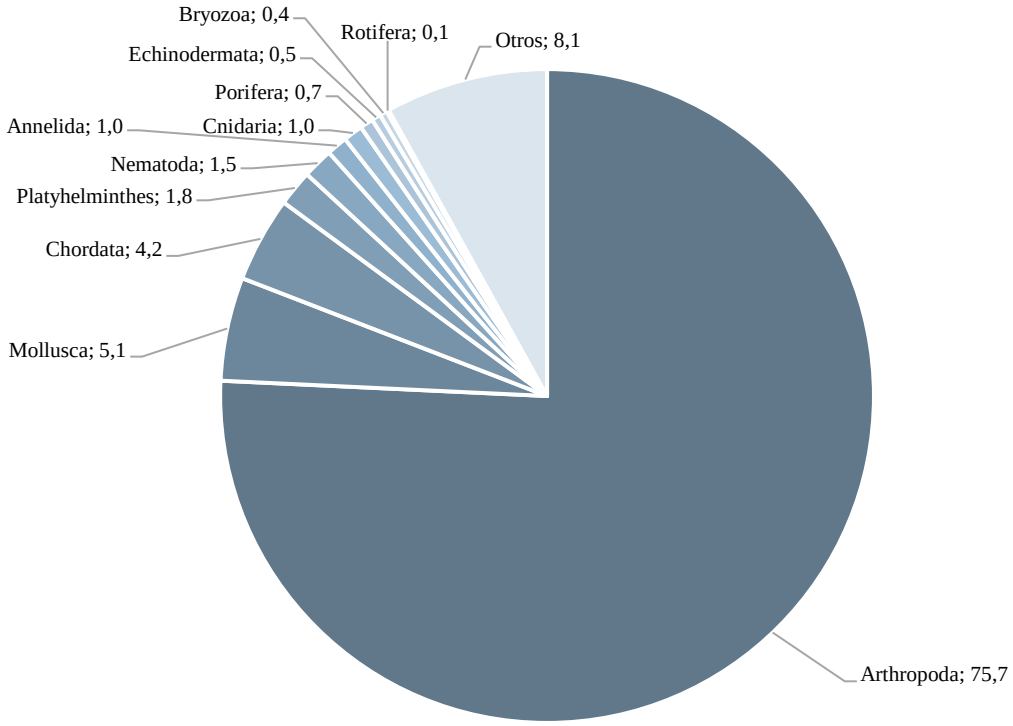


Figura 5. *Distribución en porcentaje de los filos pertenecientes al reino animal. Gráfica elaborada a partir de los datos obtenidos de Zhang (2011).*

Si realizamos una búsqueda bibliográfica en la base de datos Scopus de trabajos científicos relacionados con la aplicación de harina de insectos en dietas de acuicultura, obtenemos que el interés de esta fuente alternativa de proteína ha crecido de forma drástica en los últimos 10 años (**Figura 6**). Si clasificamos estos trabajos por países, vemos que la mayor parte de la investigación, con harina de insectos y su aplicación en

acuicultura, se ha realizado en China (364 documentos), Italia (233 documentos), EE.UU. (157 documentos), Alemania (95 documentos) y España (94 documentos).

Los insectos forman parte de la alimentación natural de muchos peces, son fuentes de proteína baratos y sostenibles. Desde el punto de vista ambiental los insectos producen menores cantidades de gases de efecto invernadero, requieren menor cantidad de agua y suelo, y poseen mejores valores del índice de conversión debido a que no requieren de energía para regular su temperatura corporal (Oonincx y de Boer, 2012; van Huis y Oonincx, 2017; Guiné et al., 2021).

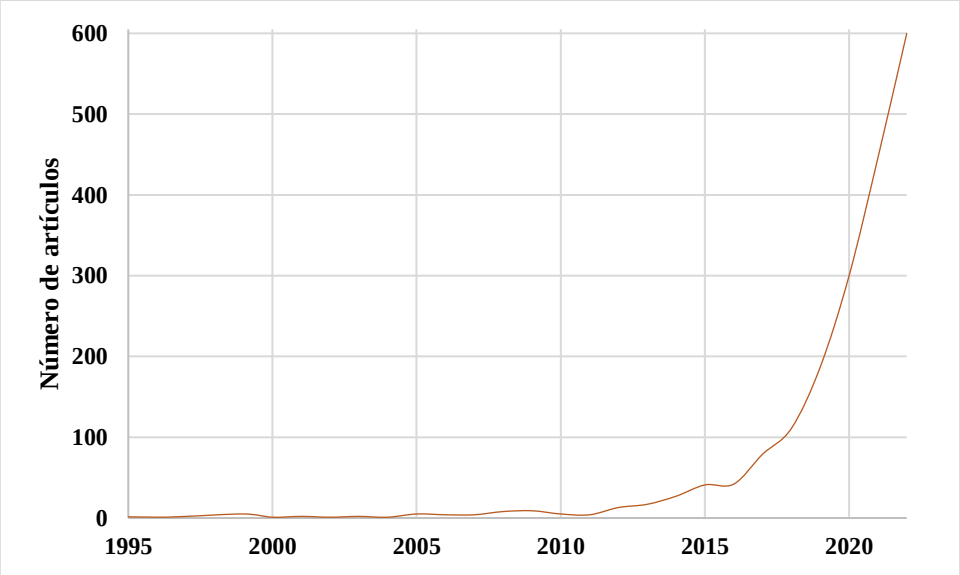


Figura 6. *Numero de artículos publicados anualmente relacionadas con la aplicación de insectos en acuicultura. Fuente: búsqueda en SCOPUS, palabras clave:* "insect meal" OR "*Tenebrio molitor*" OR "Insecta" OR "*Hermetia illucens*" OR "*Musca domestica*" OR "*Imbrasia belina*" OR "Chironomids" OR "*Gryllus bimaculatus*" OR "*Bombyx mori*" OR "Oxya hyla hyla" OR "*Zonocerus variegatus*" OR "*Zophobas morio*" OR "*Cirina butyrospermi*" OR "*Gryllodes sigillatus*" OR "*Blatta lateralis*" OR "*Oxya fuscovittata*" OR "*Acheta domesticus*" OR "*Gryllus assimilis*" OR "Termite") AND ("fish meal" OR "fishmeal") AND (fish OR shrimp) AND ("growth").

Por otro lado, cabe destacar su capacidad de transformar subproductos alimentarios de poco valor en biomasa rica en proteínas (Hem et al., 2008; Oonincx et al., 2015; Barroso et al., 2017; Spranghers et al., 2017; Barroso et al., 2019; Romero-Lorente et al., 2022). Oonincx et al. (2012) destaca que las larvas de *Tenebrio molitor* (TM) producen menores cantidades de gases de efecto invernadero y requieren de menor espacio de cría que los animales de la ganadería tradicional. Por otra parte, insectos como las larvas de *Tenebrio molitor*, Linnaeus 1758 (TM) y *Hermetia illucens,* Linnaeus 1758 (HI) aprovechan de

forma más eficiente la proteína dietaría, llegando a utilizar cerca del 50 % de esta en algunos casos (Van Huis y Oonincx, 2017).

1.2.3. Composición de los insectos

1.2.3.1. Proteína

El contenido proteico de los insectos varía mucho según la especie, el estadio en el que se encuentre, las condiciones de cría y su alimentación. En la **Tabla 1** se puede apreciar el rango en el contenido de proteína de las especies de insectos más estudiadas en acuicultura. En general, los insectos contienen una elevada proporción de proteína, pero en insectos como el grillo domestico (*Acheta domesticus*), la proteína puede llegar a suponer aproximadamente el 71 % del total del peso en seco del animal.

Tabla 1. *Valores de proteína cruda en especies de insectos y ordenes seleccionadas por su uso en acuicultura*

Especie/Orden	Estadio	% Proteína cruda
Hermetia illucens	**Larva**	36,7-47,1 %
Musca domestica	**Larva**	47,1-64,0 %
	Pupa	58,3-63,1 %
Diptera		35,9-70,1 %
Tenebrio molitor	**Larva**	20,9-52,8 %
	Pupa	53,1-54,6 %
	Adulto	60,2-66,3 %
Zophobas morio	**Larva**	43,1-46,8 %
Coleoptera		8,80-71,1 %
Bombyx mori	**Larva**	48,7-69,8 %
Lepidoptera		13,2-74,4 %
Acheta domesticus		55,0-70,8 %
Gryllus assimilis		55,5-65,5 %
Orthoptera		6,20-72,7 %

Datos extraídos de Rumpold y Schlüter (2013), Sánchez-Muros et al. (2014), Payne et al. (2016) y Alfiko et al. (2022).

En cuanto a los órdenes, los rangos varían de forma similar en todos los representantes analizados a este nivel taxonómico, donde Lepidoptera alcanza niveles cercanos al 75 % de proteína en materia seca. Este alto contenido en proteína convierte a los insectos en una fuente adecuada de proteínas para la acuicultura. Tal y como se ha indicado en los organismos unicelulares, los análisis convencionales de proteína cuantifican la cantidad de nitrógeno presente en la materia, y posteriormente este nitrógeno se traslada a proteína multiplicándolo por un factor de corrección. El factor comúnmente utilizado (6,25) proviene del porcentaje

medio de nitrógeno en proteína (16 %). Sin embargo, algunos autores consideran que el factor de corrección usado depende de la materia analizada, para evitar la sobreestimación de la proteína. Mientras Finke (2007) estima que 6,25 como factor resulta adecuado para cuantificar el contenido real de la proteína en insectos, otros autores consideran que este factor supone una sobreestimación de la proteína real (Janssen et al., 2017; Belghit et al., 2019; Boulos et al., 2020). Esta sobreestimación proviene, además de los compuestos nitrogenados no proteicos como ácidos nucleicos, fosfolípidos y compuestos nitrogenados, del nitrógeno presente en la quitina del exoesqueleto de los insectos (aspecto que será abordado con mayor profundidad en un próximo apartado). Por ello, estos autores han calculado nuevos factores de corrección para los insectos, más aceptados hoy día, que oscilan entre 4,10 y 5,33, dependiendo de la especie y etapa de desarrollo.

Respecto a la calidad de esta proteína, el perfil de aminoácidos esenciales y no esenciales analizados en diferentes insectos muestra que ninguno alcanza los valores presentes en la harina de pescado. Cierto es que el contenido en algunos aminoácidos puede ser superior en algunos insectos respecto a la harina de pescado; sin embargo, esto se da en aminoácidos puntuales y no en todo el perfil aminoacídico (Sánchez-Muros et al., 2014). Por lo general los insectos son deficientes en lisina y triptófano, y sus niveles de aminoácidos sulfurados son limitados (Rumpold y Schlüter, 2013; Makkar et al., 2014). La investigación llevada a cabo por Barroso et al. (2014) mostró que el perfil aminoacídico del orden Orthoptera era el más similar a la harina de pescado, y considerando estos datos, propusieron que la combinación de varias especies podría ser una estrategia viable a la hora de formular una harina con niveles de aminoácidos adecuados. En la **Tabla 2** se muestra la composición de aminoácidos esenciales en los dos insectos estudiados en esta tesis (TM y HI), así como la de las harinas de pescado y de soja (como alimentos de referencia).

Tabla 2. *Perfil de aminoácidos esenciales (% aminoácido/100 g proteína) en dos insectos y dos harinas usadas en acuicultura.*

	Arg	**His**	**Ile**	**Leu**	**Lys**	**Met+Cys**	**Phe**	**Thr**	**Trp**	**Val**
Hermetia illucens	5,4	3,1	4,7	6,4	6,4	2,4	**4,7**	3,8	1,0	**8,6**
Tenebrio molitor	5,6	**3,5**	**4,8**	**9,2**	6,0	2,6	4,0	3,9	0,9	6,4
Harina de pescado	6,2	2,4	4,2	7,2	**7,5**	**3,5**	3,9	**4,1**	1	4,9
Harina de soja	**7,6**	3,1	4,2	7,6	6,2	2,7	5,2	3,8	**1,4**	4,5

Datos extraídos de Alfiko et al. (2021) y Kierończyk et al. (2022). En negrita el valor máximo para cada aminoácido.

El perfil aminoacídico mostrado por TM y HI presenta niveles adecuados de la mayoría de los aminoácidos esenciales, en comparación a la harina de pescado. Además del perfil aminoacídico, otro aspecto a tener en cuenta en la calidad de la proteína de una materia prima es su digestibilidad. El perfil de aminoácidos solo representa la cantidad y distribución de aminoácidos hidrolizables en proteína; la digestibilidad es un parámetro que nos indica la bioaccesibilidad de los aminoácidos presentes en la proteína. La digestibilidad implica hidrolizar la proteína mediante acción enzimática hasta obtener los aminoácidos asimilables por el organismo. Este proceso se puede replicar *in vitro,* y usarse como aproximación de la porción bioaccesible de una proteína.

En su revisión, Rodríguez-Rodríguez et al. (2022) recogieron diversos estudios de digestibilidad proteica *in vitro* de harinas de insecto, los valores de digestibilidad variaban según la especie, etapa de desarrollo y técnica usada para medir la digestibilidad. Los valores más altos se obtuvieron para la digestibilidad analizada mediante el balance de nitrógeno inicial y final de la digestión respecto a métodos espectrofotométricos de cuantificación de aminoácidos. En las larvas de HI la digestibilidad variaba entre 60-90 %, y similarmente, la digestibilidad de TM entre 60-92 %.

1.2.3.2. Lípidos

La fracción lipídica en los insectos es el segundo mayor constituyente corporal, después de la proteína (Barroso et al., 2014; Sánchez-Muros et al., 2014). Los lípidos juegan un papel esencial en el desarrollo adecuado de los insectos. Estos son almacenados en distintas zonas en los insectos, aunque principalmente se acumulan en el cuerpo graso. Los lípidos se usan como fuente energética en un gran número de actividades fisiológicas, como periodos de ayuno, desarrollo embrionario, largos periodos de vuelo, etc. (Toprak et al., 2020).

Respecto a la cantidad y composición de los lípidos, estos variaran, del mismo modo que lo hacían las proteínas, según la especie, la etapa de desarrollo, las condiciones de cría y los alimentos ingeridos. En la **Tabla 3** se muestra la cantidad de lípidos presentes en los insectos y órdenes más comúnmente utilizados en acuicultura. Por lo general, respecto al estadio en el que se encuentran los insectos, las larvas poseen una mayor cantidad de lípidos que los adultos, ya que deben acumular reservas que usarán en los procesos complejos de la metamorfosis (Aguilar, 2021). Respecto a los niveles de lípidos por órdenes, vemos que, por lo general, los mayores niveles se dan en insectos pertenecientes al orden Coleoptera, concretamente en el estadio larvario, por tener una metamorfosis

compleja (insectos holometábolos). Por el contrario, los niveles más bajos estudiados aparecen en el orden Orthoptera, ya que al ser hemimetábolos (metamorfosis simple), las ninfas son similares a los adultos.

Tabla 3. *Valores de grasa bruta en especies y ordenes seleccionadas por su uso en acuicultura.*

Especie/Orden	Estadio	% Grasa bruta
Hermetia illucens	**Larva**	26,0-34,8 %
Musca domestica	**Larva**	6,70-25,3 %
	Pupa	15,5-15,8 %
Diptera		6,70-34,8 %
Tenebrio molitor	**Larva**	32,8-43,1 %
	Pupa	30,8-36,7 %
	Adulto	14,9-18,4 %
Zophobas morio	**Larva**	40,8-43,6 %
Coleoptera		0,66-69,8 %
Bombyx mori	**Larva**	8,09-25,7 %
Lepidoptera		5,25-77,2 %
Acheta domesticus		9,80-24,0 %
Gryllus assimilis		11,8-21,8 %
Orthoptera		2,49-53,1 %

Datos extraídos de Rumpold y Schlüter (2013), Sánchez-Muros et al. (2014), Payne et al. (2016), Soares Araújo et al., (2019) y Alfiko et al. (2021).

Los ácidos grasos representan el componente principal de la fracción lipídica (Barroso et al., 2014; Sánchez-Muros et al., 2014; Nowak et al., 2016). De forma general, la fracción de ácidos grasos saturados es menor que los niveles de ácidos grasos insaturados (Gonçalves dos Santos Aguilar, 2021). La composición de ácidos grasos para los distintos órdenes muestra que en Diptera, por lo general, son mayoritarios los ácidos grasos monoinsaturados (MUFA), seguidos de ácidos grasos poliinsaturados (PUFA) y ácidos grasos saturados (SFA). En Lepidoptera y Orthoptera, el grupo de ácidos grasos dominantes son los PUFA, seguidos de SFA y MUFA. Coleoptera presenta una mayor cantidad de SFA que del resto de grupos de ácidos grasos (Rumpold y Schlüter, 2013). En la **Tabla 4** podemos observar la composición de los ácidos grasos de los insectos tratados en esta tesis. Por lo general, en HI los ácidos grasos más predominantes son los ácidos grasos saturados, a diferencia del aceite de pescado y el aceite de soja. La mayor aportación a este grupo lo hace el ácido láurico (12:0), que puede llegar a suponer más del 60 % del total de ácidos grasos. En el resto de los insectos, los niveles de ácido láurico

no suelen superar el 1 %. En TM se observa como lo ácidos grasos monoinsaturados son los que se encuentran en mayor proporción, especialmente el ácido oleico (18:1*n*9), mientras que en HI este grupo lo componen principalmente el ácido palmitoleico (16:1*n*7) y el ácido oleico.

Tabla 4. *Ácidos grasos (% ácido graso/ácidos grasos totales) en dos insectos y dos harinas utilizadas habitualmente en acuicultura.*

	SFA	MUFA	PUFA
Hermetia illucens	51,30-81,81	8,14-29,0	6,68-23,3
Tenebrio molitor	8,00-33,7	13,4-59,9	9,32-33,8
Aceite de pescado	23,32	19,33	19,14
Aceite de soja	14,56	21,06	58,73

Datos obtenidos de Nogales-Merida et al. (2019). SFA: ácidos grasos saturados; MUFA: ácidos grasos monoinsaturados; PUFA: ácidos grasos poliinsaturados.

En los insectos terrestres, los ácidos grasos poliinsaturados son el grupo que menos aporta al total de ácidos grasos. Desde el punto de vista de la acuicultura, los ácidos grasos EPA y DHA son especialmente importantes. Estos ácidos grasos no aparecen en insectos terrestres; solo se ha detectado la presencia de EPA en insectos acuáticos, pero no DHA (Sánchez-Muros et al., 2014). Aunque los insectos terrestres poseen las enzimas necesarias para sintetizar EPA, lo hacen en cantidades muy bajas, destinando su producción a la síntesis de eicosanoides (Stanley y Kim, 2014).

Al contrario de lo que ocurre con el porcentaje de aminoácidos en las proteínas, la composición de ácidos grasos en los lípidos de los insectos está sujeta en gran medida a la alimentación, por lo tanto, es posible modificar su perfil de ácidos grasos mediante la introducción de fuentes alimentarias con perfiles de ácidos grasos más valiosos (Barroso et al., 2019).

1.2.3.3. Quitina

La quitina (**Figura 7**) es un amino polisacárido presente principalmente en el exosqueleto de lo artrópodos, aunque también se puede encontrar en la pared celular de los hongos y en nematodos. Está formada mayormente por monómeros de *N*-acetilglucosamina, aunque también forma parte del polímero el monómero N-glucosamina. Estos monómeros se encuentran unidos por enlaces *β*-(1-4)-glucosídicos (Rinaudo, 2006). De forma natural, la quitina raramente se encuentra pura, sino que se encuentra en combinación con otros compuestos. En el caso de los insectos, la quitina se encuentra en combinación de proteína, lípidos y otros tipos de compuestos. En el caso de los crustáceos, la quitina se encuentra en

combinación con proteínas y minerales, principalmente calcio. Estas estructuras con calcio también se dan en insectos como HI (Finke, 2007).

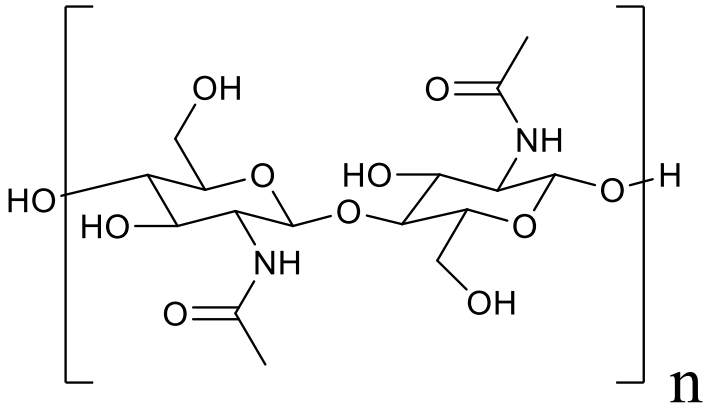


Figura 7. *Estructura de la quitina.*

Durante el proceso de esclerotización de la cutícula, las fibras de quitina se unen a proteínas cuticulares endureciéndolas. Estas reacciones se llevan a cabo por quinonas que reaccionan con grupos funcionales presentes en las proteínas propias de la cutícula, que se consideran proteínas no digeribles. La proporción de estas proteínas respecto a la quitina modifica la naturaleza mecánica del exosqueleto, incrementando su dureza al aumentar la proporción de proteína respecto a la quitina (Jonas-Levi y Martinez, 2017; Nogales-Merida et al., 2019). El contenido en quitina en insectos va desde el 2,5 % hasta el 36,6 % (Abidin et al., 2020).

El complejo quitina-escleroproteína se considera como materia no digerible, ya que la presencia de las enzimas encargadas de hidrolizar estas proteínas sólo se encuentra en organismos cuyos hábitos alimentarios incluyen insectos o crustáceos (Ikeda et al., 2017; Holen et al., 2022). Aunque la ingesta moderada de quitina por parte de peces puede suponer una mejora en parámetros de crecimiento, una mayor respuesta inmune y actividad prebiótica, cantidades más altas pueden desencadenar una respuesta alérgica por parte del pez y afectar al crecimiento (Lock et al., 2018; Tran et al., 2022).

1.2.4. Normativa que regula la cría y uso como alimento de los insectos

En la actualidad, la legislación que regula la cría de insectos y su uso como alimento es bastante compleja y en continuo cambio, ya que debe adaptarse a un mercado que evoluciona día a día.

Con el objetivo de desarrollar un sistema alimentario más sostenible, la Unión Europea está buscando nuevas fuentes de proteína ("Nuevos alimentos"), entre los que se encontrarían los insectos. La Unión Europea entiende como "Nuevo alimento" cualquier alimento que no hubiese sido consumido normalmente antes del 15 de mayo de 1997.

1.2.4.1. Uso de insectos como PAT (proteína animal transformada) en la alimentación de animales productores de alimentos

Actualmente, el uso de insectos como proteína animal transformada se encuentra regulado en la Unión Europea por el Reglamento (CE) 2017/893 de la Comisión Europea. En este reglamento se establecen una serie de características a cumplir por parte de las fuentes de alimento para poder ser usadas como pienso en la alimentación de animales productores de alimentos. Del mismo modo, en el reglamento se recoge que estos insectos solo pueden usarse en la alimentación en acuicultura, aves de corral y porcino. Esta misma regulación prohíbe la alimentación de insectos con carne o pescado. Sin embargo, sí se permite la alimentación de los insectos con productos derivados de animales como la harina de pescado, hemoderivados, proteínas hidrolizadas de no rumiantes, productos lácteos, huevos, etc.

Las especies de las que se pueden obtener estas PAT están reguladas en el Anexo II del Reglamento (CE) 893/2017. Así́, la PAT destinada a la producción de piensos para animales de granja, solo podrá́ obtenerse de las siguientes especies de insectos:

- *Alphitobius diaperinus*.
- *Gryllus assimilis*.
- *Acheta domesticus*.
- *Gryllodes sigillatus*.
- *Tenebrio molitor*.
- *Musca domestica*.
- *Hermetia illucens*.

1.2.4.2. Uso de los insectos en alimentación humana

En enero del 2018, el Reglamento (CE) 2015/2283 concluye que los insectos y sus partes (alas, patas y cabeza) pasan todas las pruebas necesarias para que la Unión Europea los considerase alimentos. Y con el objeto de reforzar la seguridad jurídica se incorpora la incorpora la definición de "insecto de granja", como el animal de granja, a tenor de la definición recogida en el Reglamento (CE) 1069/2009.

Este Reglamento prevé dos tipos de procedimientos para incorporar a los insectos como alimento:

- procedimiento de **solicitud de autorización** de nuevos alimentos.
- procedimiento de notificación para **alimentos tradicionales de terceros países**, que se basará en el historial de uso alimentario seguro en un tercer país, de manera que tales alimentos deben haber sido consumidos en al menos un tercer país durante por lo menos veinticinco años como parte de la dieta habitual de un número significativo de personas.

En consecuencia, cualquier operador que quiera comercializar insectos para alimentación humana en la Unión Europea, debe presentar una solicitud de autorización o de notificación, en base a uno de los dos procedimientos. Una vez que la Comisión Europea lo incluya en la lista de la Unión, tal y como prevé el Reglamento, se podrá iniciar su comercialización.

1.2.4.2.1. Medidas transitorias

Para los insectos que se comercialicen en la Unión Europea se prevé un periodo transitorio en el que se podrán seguir comercializando hasta que se adopte una decisión de conformidad con el procedimiento de autorización de nuevos alimentos o con el procedimiento de autorización de alimento tradicional de terceros países.

Actualmente, y aunque estamos fuera del periodo fijado en el Reglamento (UE) 2015/2283, las medidas transitorias se mantienen hasta que se tome una decisión al respecto sobre su inclusión o no en la lista de la Unión Europea.

Entre las especies de insectos, que actualmente pueden estar en el mercado europeo por estar acogidos a las medidas transitorias establecidas en dicho Reglamento, en tanto se llega a una decisión sobre su inclusión o no en la lista de la Unión (Reglamento de Ejecución (UE) 2017/2470), encontramos:

- *Apis mellifera*.
- *Alphitobius diaperinus*.
- *Acheta domesticus*.
- *Gryllodes sigillatus*.
- *Tenebrio molitor*.
- *Schistocerca gregaria*.
- *Locusta migratoria*.

1.2.4.2.2. Insectos ya autorizados para su comercialización en la UE

Respecto a la seguridad alimentaria, la Autoridad Europea de Seguridad Alimentaria (EFSA) emitió un comunicado en 2015 sobre la necesidad de ampliar las investigaciones referentes a los potenciales riesgos biológicos y químicos de los insectos, tanto los usados en alimentación animal como humana. Y después de una rigurosa evaluación científica realizada por esta Autoridad, los Estados miembros ya han autorizado a introducir diversas especies de insectos en el mercado de la Unión Europea:

- Mayo 2021: se aprueba el primer insecto como alimento, *Tenebrio molitor* (Reglamento de Ejecución (UE) 2021/882 y Reglamento de Ejecución (UE) 2022/169).
- Noviembre 2021: se aprueba el segundo insecto como alimento, *Locusta migratoria* (Reglamento de Ejecución (UE) 2021/1975).
- Febrero 2022: se aprueba el tercer insecto como alimento, *Acheta domesticus* (Reglamento de Ejecución (UE) 2022/188).
- En enero de 2023 (Reglamento de Ejecución (UE) 2023/58) se autoriza la comercialización de las formas congelada, en pasta, desecada y en polvo de las larvas de *Alphitobius diaperinus* como nuevo alimento.

1.2.5. *Hermetia illucens* y *Tenebrio molitor*

Estas dos especies de insectos son las más estudiadas y empleadas actualmente en la acuicultura a nivel mundial.

Los contenidos y composición de la proteína y los lípidos en larvas de HI y TM se han descrito en las secciones anteriores. Y también se ha mencionado que la composición, aunque sigue cierto patrón, puede modificarse en cierto grado con la alimentación. Por ello, para obtener una biomasa de insecto rica en proteína y lípidos de calidad, es necesario conocer las condiciones de cría, hábitos alimentarios y etapas de desarrollo de estos insectos.

1.2.5.1. *Hermetia illucens* (HI)

Hermetia illucens, también conocida como mosca soldado-negra (**Figura 8**), es un insecto procedente originalmente de América, más específicamente de zonas subtropicales, aunque actualmente se encuentra distribuida en las todas regiones del mundo con climas adecuados para su reproducción o en micro-ganaderías con control climático.

Reino	Animalia
Filo	Arthropoda
Clase	Insecta
Orden	Diptera
Familia	Stratiomyidae
Género	*Hermetia*
Especie	*H. illucens*

Figura 8. Taxonomía *e imagen de larva de H. illucens.*

En Europa se describió por primera vez en Malta en 1926, mientras que en España el primer registro data del año 1954 (Martínez-Sánchez et al., 2010). HI es un insecto holometábolo (con metamorfosis compleja). Las larvas de esta especie son saprófagas y fotofóbicas, y su desarrollo y alimentación ocurre en materia orgánica en descomposición. Presentan mandíbulas fuertes que facilitan la ingesta de alimentos y movilidad. El color beige que adoptan durante su estadio larvario se torna a marrón oscuro al entrar en estadio de prepupa. Los adultos pueden alcanzan una longitud de 13-20 mm, poseen dos antenas y un par de alas funcionales. Los individuos hembra adultos suelen tener un tamaño superior al de los machos (Tomberlin y Sheppard, 2002).

Las hembras depositan alrededor de 320-1000 huevos en hileras cerca de fuentes de alimento, y poco después de la oviposición mueren (Tomberlin y Sheppard, 2002). Estos huevos poseen forma ovalada y miden aproximadamente 1 mm de largo. La eclosión se da tras alrededor de 4 días de incubación a una temperatura de 25-29 ºC, aunque puede reducirse el tiempo de incubación con temperaturas más altas (Anderson, 2000). La duración del estado larvario se extiende entre cuatro semanas y cinco meses, dependiendo de la disponibilidad de alimento y la temperatura. El estadio larvario se puede separar en cinco etapas de desarrollo, aunque solamente la última (prepupa) es fácilmente diferenciable, por su característico color marrón oscuro. La metamorfosis se prolonga aproximadamente dos semanas, y las pupas suelen medir aproximadamente 12-25 mm. Tras la metamorfosis, la reproducción se lleva a cabo tras los dos primeros días y su esperanza de vida se encuentra entre 5-14 días, dependiendo de las reservas energéticas y la disponibilidad de agua (Tomberlin et al., 2009).

Debido a su capacidad de alimentarse a partir de diversas materias orgánicas, se han llevado a cabo numerosos estudios donde se han evaluado los efectos de estas dietas en

el rendimiento y composición nutritiva de HI. Entre las diferentes dietas encontramos desperdicios de comida humana (Spranghers et al., 2017; Lalander et al., 2020; Pang et al., 2020), residuos vegetales (Hem et al., 2008; Oonincx et al., 2015; Spranghers et al., 2017), aceites vegetales (Oonincx et al., 2020; Georgescu et al., 2022) y pescado (St-Hilaire et al., 2007; Barroso et al., 2019).

El uso de la harina de HI en acuicultura está muy extendido, y el número de revisiones sobre el uso de este insecto en acuicultura son numerosas (Henry et al., 2015; Hua et al., 2019; Hua, 2021; Gasco et al., 2023; Maulu et al., 2022; Mohan et al., 2022; Tran et al., 2022).

1.2.5.2. *Tenebrio molitor* (TM)

Tenebrio molitor, también conocido como gusano de la harina (**Figura 9**), es una especie que se encuentra distribuida por todo el mundo. Esta especie se alimenta de productos farináceos, y es considerada una plaga de baja importancia. Estos insectos son holometábolos, las larvas poseen seis patas y mandíbula. Su coloración es amarillenta durante todo el estadio larvario. Los adultos tras la metamorfosis poseen un color blanquecino, aunque, al finalizar su desarrollo adulto, torna a una coloración marrón rojizo. Esta especie puede alimentarse de cualquier derivado de cereal y vegetales (Allen et al., 2012; Siemianowska et al., 2013); además, poseen la habilidad de seleccionar la ingesta de alimentos específicos, con el fin de calibrar la ingesta adecuada de nutrientes (Morales-Ramos et al., 2011; Rho y Lee, 2016).

Reino	Animalia
Filo	Arthropoda
Clase	Insecta
Orden	Coleoptera
Familia	Tenebrionidae
Género	*Tenebrio*
Especie	*T. molitor*

Figura 9. Taxonomía *e imagen de larva H. illucens.*

Las hembras depositan entre 400-500 huevos de forma individual o en pequeños grupos. Tras la deposición de los huevos, la eclosión se produce después de 10-12 días a una

temperatura de 18-20 ºC (Ghaly et al., 2009), aunque la temperatura juega un papel relevante en el tiempo de eclosión. Las larvas pasan por un gran número de etapas de desarrollo antes de la metamorfosis, y pueden llegar a medir longitudinalmente 9-28 mm (Ribeiro et al., 2018). La etapa larvaria puede llegar a durar entre 57-600 días, aunque el tiempo medio se sitúa entre 100-200 días. La metamorfosis dura entre 6 y 20 días. Los adultos emergen de la pupa y pueden llegar a medir entre 12-16 mm al alcanzar la madurez, característica que alcanzan a los 3 días de emerger. La esperanza de vida de los adultos suele rondar los 16-170 días (Cotton, 1927; Finke et al., 2002; Ghaly et al., 2009).

Los ensayos de alimentación llevados a cabo con TM han sido principalmente realizados con dietas basadas en residuos vegetales (Ramos-Elorduy et al., 2002; Oonincx et al., 2015; van Broekhoven et al., 2015; Mancini et al., 2019; Harsányi et al., 2020; Bordiean et al., 2022), aunque también se han llevado a cabo experiencias con descartes de pesca (Romero-Lorente et al., 2022).

Junto a HI, TM es una de las especies con mayor recorrido en investigación en acuicultura (Henry et al., 2015; Hua et al., 2019; Hua, 2020; Gasco et al., 2023; Shafique et al., 2021; Soares de Lima et al., 2021; Maulu et al., 2022; Tran et al., 2022).

1.3. Nutrición en acuicultura

La nutrición en acuicultura es similar a la nutrición de animales terrestres en cuanto al tipo de macromoléculas y necesidades energéticas. Aunque hay que destacar ciertas diferencias entre los requerimientos de nutrientes en peces y animales terrestres: (1) las necesidades energéticas son inferiores en peces que en animales, debido a que no invierten energía en mantener su temperatura corporal; (2) las necesidades de ácidos grasos omega-3 son superiores en los peces; (3) los peces poseen la capacidad de absorber minerales directamente del agua; y (4) la mayoría de peces son incapaces de sintetizar vitamina C, por lo que dependen de las fuentes exógenas (Lovell, 1998).

Las necesidades entre especies de peces varían. Las necesidades proteicas y lipídicas, ácidos grasos esenciales y la utilización de carbohidratos están regidas por la temperatura del agua, el nivel trófico y el hábitat, principalmente (NRC, 2011).

1.3.1. Proceso de digestión

La anatomía del aparato digestivo de los peces se compone de boca, faringe, esófago y estómago; a continuación, se encuentran los ciegos pilóricos, el intestino y, finalmente, el recto. Las diferencias morfológicas de cada uno de estos órganos se encuentran sujetos

principalmente a los hábitos alimentarios de estos organismos. Tras la eclosión, los peces presentan un aparato digestivo primitivo sin estomago desarrollado. Durante el estado larval y juvenil, el tracto digestivo se desarrolla y aparecen estructuras más complejas y funcionales. Por lo general, algunos peces herbívoros no llegan a desarrollar un estómago verdadero, incluso en su etapa adulta, y presentan un intestino muy largo, mientras que los peces carnívoros presentan estómago bien definido, ciegos pilóricos y un intestino bastante más corto que en las especies herbívoras (Halver y Hardy, 2002; NRC, 2011).

La hidrólisis de los macronutrientes se da principalmente en el estómago y en el intestino. En el estómago, la digestión se lleva a cabo por medio de la secreción del jugo gástrico. La pepsina presente en este jugo es una endopeptidasa que se encarga de hidrolizar enlaces peptídicos en proteínas adyacentes a aminoácidos con un sustituyente aromático (Halver y Hardy, 2002; NRC, 2011). Esta enzima se secreta en forma de pespsinógeno y se ve activada por la presencia de ácido clorhídrico. Algunos peces con alimentación basada en crustáceos e insectos presentan actividad quitinolítica, con capacidad de hidrolizar los enlaces glucosídicos de la quitina presente en el exoesqueleto de los artrópodos (Gutowska y Drazen, 2004; Ikeda et al., 2017).

Los nutrientes parcialmente digeridos llegan al intestino en forma de quimo, donde se secretan la bilis y el jugo pancreático. El jugo pancreático está compuesto por diversas enzimas encargadas de hidrolizar los nutrientes para facilitar su absorción a través de la mucosa intestinal. Entre las enzimas proteasas alcalinas encontramos tripsina, quimotripsina, elastasas, carboxipeptidasas y aminopeptidasas; estás se encargan de hidrolizar enlaces peptídicos con diferente especificidad. Las lipasas son enzimas encargadas de hidrolizar los acilgliceroles; esta hidrólisis se encuentra facilitada por la acción emulsificante de la bilis y componentes presentes en el alimento. Los carbohidratos como el almidón son hidrolizados por acción de la α-amilasa, produciendo dextrinas, maltotriosas y maltosa (Halver y Hardy, 2002; NRC, 2011). Finalmente, en la mucosa intestinal se encuentran otra serie de enzimas, como las peptidasas; estas se encargan de hidrolizar lo péptidos en aminoácidos, como previo paso a su absorción y transporte.

Estas enzimas están sujetas a variaciones, y responden a cambios alimentarios adaptando su actividad (Chakrabarti et al., 1995; Moyano et al., 1996; Hidalgo et al., 1999; Gelman et al., 2008; Santigosa et al., 2008; Falcón-Hidalgo et al., 2011; Gioda et al., 2017). En

general, la acción de la α-amilasa y proteasa alcalina es mayor en los peces herbívoros y omnívoros que en los peces carnívoros (Hidalgo et al., 1999).

1.3.2. Necesidades energéticas

Las necesidades energéticas se cubren con la energía liberada tras la oxidación metabólica de carbohidratos, lípidos y aminoácidos. Estas necesidades son cubiertas con el alimento, y es utilizada para mantener procesos metabólicos necesarios para el mantenimiento y crecimiento del animal. La principal diferencia que encontramos entre peces y animales de producción terrestre es la capacidad de los primeros de eliminar, a través de las branquias, el amoniaco proveniente del metabolismo proteico, sin necesidad de usar energía en transformar el amoniaco en urea o derivados, como ocurre en los animales de producción (Halver y Hardy, 2002; NRC, 2011). Además, al ser poiquilotermos, no necesitan utilizar energía en el mantenimiento de la temperatura corporal y requieren menos energía en gastos de movimiento.

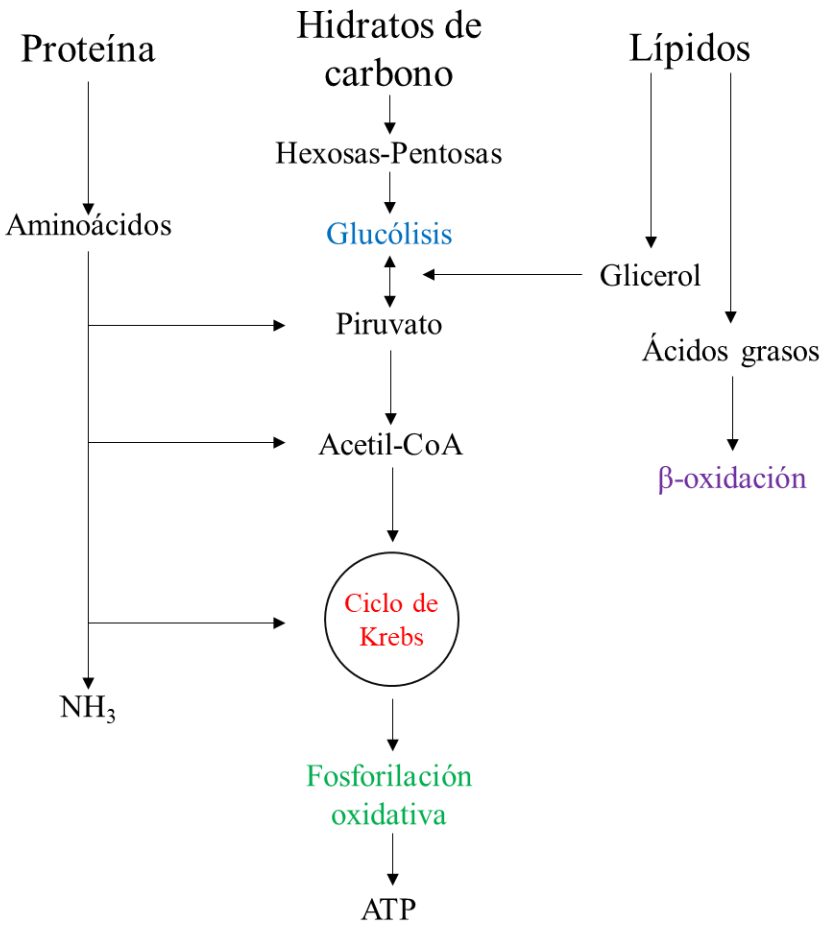


Figura 10. *Esquema de las principales rutas metabólicas*.

Como se ha comentado anteriormente, los peces obtienen la energía a través de la oxidación tanto de lípidos y carbohidratos, como de aminoácidos. Los aminoácidos, antes de su uso como fuente energética, deben de someterse a un proceso de desaminación previo. En este paso, los aminoácidos son convertidos en un α-cetoácido y posteriormente derivados a distintos pasos del ciclo de los ácidos tricarboxilicos o a gluconeogénesis, dependiendo del aminoácido de partida (**Figura 10**). En el caso de los lípidos, estos se deben descomponer en glicerol, el cual posteriormente se convierte en piruvato y ácidos grasos, los cuales, mediante β-oxidación, se transforman en acetil-CoA. Los hidratos de carbono son metabolizados a piruvato y acetil-CoA para, posteriormente, ser incorporados en el ciclo de los ácidos tricarboxilicos. También se encuentra presente en las rutas la vía de las pentosas fosfato.

Debido a que el desarrollo evolutivo de los peces ha sido en un entorno acuático, donde no abundan los carbohidratos, las principales fuentes energéticas son las proteínas y los lípidos, mientras que los carbohidratos son utilizados menos eficientemente. La capacidad de asimilar carbohidratos es dependiente, principalmente, de los hábitos alimenticios de los peces. Así, peces de agua dulce toleran y asimilan de manera más eficiente los carbohidratos que los peces marinos. Aunque la digestibilidad de los carbohidratos, por lo general, es inferior a los animales terrestres, los peces cuentan con cierta capacidad, dependiendo de la especie, de utilizarlos metabólicamente.

Aunque los peces no tienen necesidades específicas de hidratos de carbono, hay tejidos, como cerebro, gónadas y sangre (eritrocitos) que solo pueden usar la glucosa como sustrato energético, la cual los peces la obtienen principalmente a partir de las proteínas, vía gluconeogénesis. En cuanto a los lípidos, las principales diferencias respecto a animales terrestres las encontramos en la composición, destacando en los peces la presencia de *n*-3 LCPUFA, sobresaliendo EPA y DHA (Halver y Hardy, 2002; NRC, 2011).

1.3.3. Necesidades proteicas

Las proteínas son un componente esencial en el organismo de cualquier ser vivo. Estas macromoléculas están formadas por aminoácidos unidos por enlaces peptídicos, y juegan un papel esencial en el metabolismo y construcción de tejidos. En animales en crecimiento, la síntesis de proteína supera a su degradación, lo que hace que la proteína sea el componente más relevante en la ganancia de peso (Dumas et al., 2007). Los requerimientos de proteína responden principalmente a las necesidades de aminoácidos

esenciales y no esenciales para la síntesis de proteínas, o su uso como fuente energética. Estás necesidades proteicas se ven sujetas a varios factores, entre los que destacan los hábitos alimentarios del pez; los peces carnívoros requieren una mayor cantidad de proteína que los peces omnívoros o herbívoros (Tacon y Metian, 2008; Bowyer et al., 2013), aunque estos últimos presentan una menor eficiencia a la hora de retener la proteína (NRC, 2011). El peso del pez también juega un papel relevante en las necesidades proteicas en la dieta, al aumentar su edad los requerimientos de proteína disminuyen. No obstante, en el estudio llevado a cabo por Oliva-Teles et al. (2020), en el que revisaron las necesidades proteicas en peces obtenidas de un amplio número de ensayos, concluyeron que la relación entre el peso y la necesidad proteica no siempre sigue el mismo patrón, variando entre especies. Otros factores, como la temperatura y la salinidad, no ocasionan un efecto tan marcado, como los factores anteriores, siempre y cuando estos factores se encuentren entre rangos adecuados (NRC, 2011).

La composición de aminoácidos de la proteína es uno de los factores, junto a la digestibilidad, más importante para evaluar la calidad proteica de un alimento. Los aminoácidos se dividen en esenciales y no esenciales; está división proviene de la capacidad del organismo de sintetizar estos aminoácidos internamente (no esenciales) o no (esenciales), por lo que estos últimos deben obtenerse a través de la dieta. La mayoría de los animales requieren obtener a través de la dieta los mismos diez aminoácidos esenciales (arginina, histidina, isoleucina, leucina, lisina, metionina, fenilalanina, treonina, triptófano y valina). Los aminoácidos se destinarán, una vez absorbidos, a la síntesis de proteína, mantenimiento y catabolismo (Halver y Hardy, 2002; NRC, 2011).

1.3.3.1. Metabolismo proteico

La síntesis y degradación de proteínas corporales es un proceso continuo que permite la renovación y reparación de las proteínas que forman parte de estructuras corporales. La síntesis de proteínas requiere de la presencia de los aminoácidos necesarios para su síntesis. El proceso de síntesis se inicia con la activación de los aminoácidos por medio de una molécula de ATP y su posterior unión a ARN; dicha unión se encuentra catalizada por la acetil-t-ARN sintetasa. En los ribosomas continúa la síntesis, obteniendo la información de la secuencia de aminoácidos necesaria por medio del ARNm. La síntesis proteica se da en distintos tejidos, aunque los órganos más activos en este sentido son hígado, branquias, intestino, riñón y bazo. La temperatura, oxigeno, salinidad, ayuno y

un perfil aminoacídico desbalanceado, afectan en mayor o menor medida a la síntesis proteica en peces (Sánchez-Muros, 1990).

1.3.3.2. Catabolismo aminoacídico

Como se ha comentado anteriormente, los peces usan los aminoácidos obtenidos de las proteínas como principal fuente energética a través de varios procesos metabólicos, denominados catabolismo de aminoácidos (**Figura 11**). Existen diferentes tipos de catabolismo de aminoácidos; por un lado, encontramos el catabolismo inevitable y, por otro, el catabolismo asociado a la ingesta de aminoácidos en cantidades superiores a los requeridos. El catabolismo inevitable ocurre cuando la energía no es limitante para la síntesis de proteína; este proceso se denomina inevitable debido a la presencia de procesos metabólicos que no pueden ser inactivados (Moughan, 1995; de Lange et al., 2001).

Este proceso es constante siempre que la ingesta de aminoácidos se encuentra entre un 70-100 % de los requeridos, y puede representar entre un 20-40 % de los aminoácidos digeridos (NRC, 2011). El catabolismo asociado al exceso de aminoácidos se refiere al proceso de catabolismo dado cuando la cantidad de aminoácidos supera la cantidad destinada al resto de procesos, o cuando la dieta es deficiente en uno o varios aminoácidos esenciales, limitando la síntesis proteica, y aumentando el destino catabólico de los aminoácidos (Halver y Hardy, 2002; NRC, 2011).

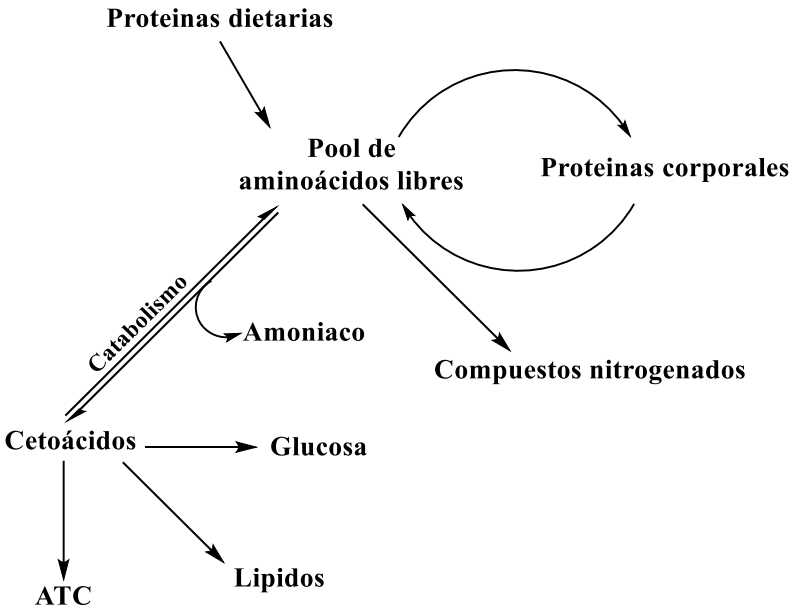


Figura 11. *Esquema del metabolismo proteico.*

El catabolismo de aminoácidos consta de dos etapas; un primer paso consiste en la desaminación directa o indirecta y la formación de un cetoácido que puede entrar en el ciclo de los ácidos tricarboxilicos. La segunda etapa consiste en la oxidación del compuesto intermediario para dar dióxido de carbono y agua. El amonio derivado del proceso de desaminación es mayormente excretado a través de las branquias (Sánchez-Muros, 1990). En la desaminación directa, la eliminación del grupo amino del aminoácido se lleva a cabo a través de enzimas como amino-oxidasa y glutamato deshidrogenasa (EC 1.4.1.2., GDH), siendo esta última la más relevante (Ballantyne, 2001). La desaminación indirecta se lleva a cabo principalmente a través de la transferencia del grupo amino a un cetoácido, que es desaminado posteriormente. Esta reacción se encuentra catalizada por transaminasas, entre las que destacan principalmente alanina transaminasa (EC 2.6.1.2., ALT) y aspartato transaminasa (EC 2.6.1.1., AST) (Walton y Cowey, 1982). Las transaminasas presentan una mayor concentración en el hígado, aunque también aparecen en otros órganos. En definitiva, las reacciones del catabolismo aminoacídico se encuentran principalmente catalizadas por ALT, AST y GDH, por lo que las variaciones en la actividad y la afinidad de estas enzimas por el sustrato determinan la utilización de la proteína dietaría (Sánchez-Muros et al., 1998; Bibiano-Melo et al., 2006; Furné et al., 2016).

1.3.3.2.1. Enzimas del catabolismo aminoacídico

La Glutamato deshidrogenasa cataliza la reacción reversible de desaminación del glutamato para dar lugar a α-cetoglutarato, amonio y NADPH (**Figura 12**). Esta reacción es de localización intramitocondrial. Está enzima requiere de NADP como cofactor y se ve inhibida por ATP y GTP, mientras que es estimulada por ADP y AMP.

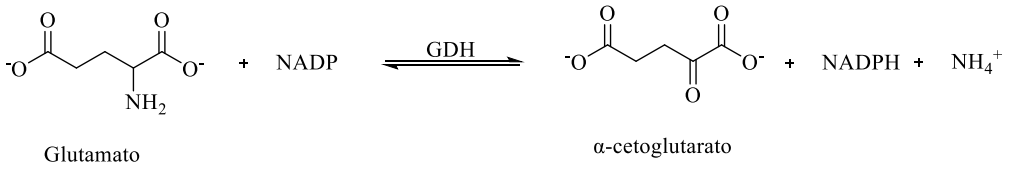


Figura 12. *Esquema de la reacción de desaminación catalizada por GDH.*

Alanina y Aspartato aminotransferasas son enzimas que catalizan la transferencia reversible del grupo amino presente en los aminoácidos alanina y aspartato hacía α-cetoglutarato, para dar lugar a piruvato u oxal-acetato y glutamato. Este glutamato es

posteriormente desaminado por acción de la GDH, rindiendo α-cetoglutarato y NH_3 (**Figura 13**). Esta reacción ocurre principalmente en el citoplasma celular. Para su funcionamiento, estas enzimas requieren de un cofactor (piridoxal fosfato) para el transporte del grupo amino, y de NADH. Estas enzimas, junto con GDH, son las principales enzimas encargadas de llevar a cabo la transdesaminación en el catabolismo aminoacídico.

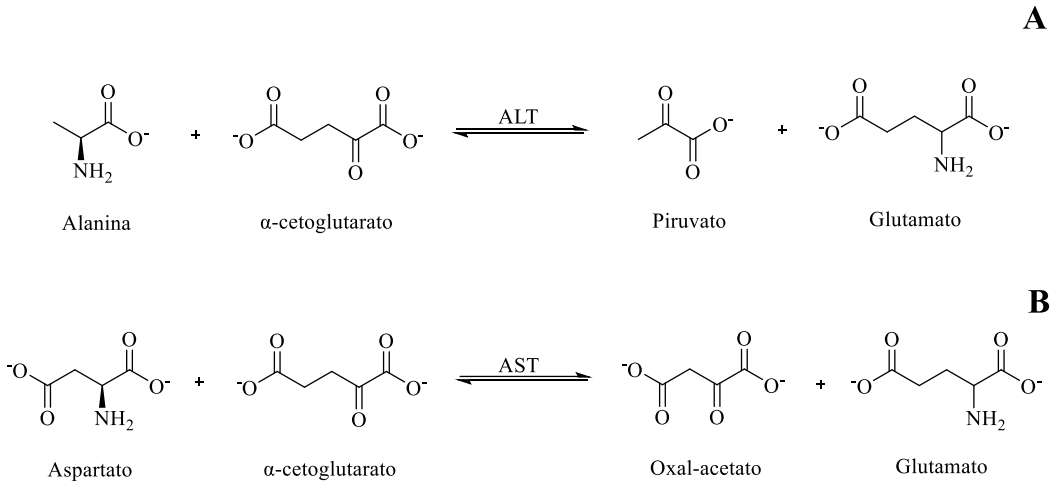


Figura 13. *Esquema de las reacciones catalizadas por ALT (A) y AST (B).*

La actividad de estas enzimas sufre modificaciones en respuesta a alteraciones dietarías. En una dieta con alto contenido proteico, ensayada en trucha arcoíris, ALT y GDH mostraron una mayor actividad respecto a la dieta control, indicando que la ingesta de dietas con alto contenido proteico propició un aumento en la síntesis de está enzima en hígado, en respuesta a un aumento en la concentración de aminoácidos. La afinidad por el sustrato para ALT y GDH no varió significativamente respecto a los animales alimentados con la dieta control (Sánchez-Muros et al., 1998). Similares resultados se obtuvieron en *Rhamdia quelen* alimentada con dietas compuestas por distintos niveles de proteína; las actividades de ALT, AST y GDH aumentaron al aumentar el porcentaje de proteína en la dieta (Bibiano-Melo et al., 2006). Dean et al. (1986) observaron para *Ictalurus punctatus* que la cantidad de proteína en dieta aumenta la actividad de ALT, AST y GDH, mientras que la calidad proteica aumentaba la actividad de AST y la actividad de GDH disminuía. En trucha arcoíris y *Acipenser naccarii*, el ayuno durante un largo periodo dio lugar a un aumento de ALT y AST en trucha arcoíris durante los

primeros 10-15 días, si bien, a continuación, la actividad se redujo drásticamente hasta alcanzar la misma actividad que al comienzo del experimento. En cambio, para *A. naccarii*, la actividad de ALT disminuyó continuamente durante todo el periodo de ayuno, mientras que la actividad de AST mostró un aumento en su actividad en los primeros 10-15 días, para posteriormente decrecer. La actividad de GDH no se vio afectada significativamente en ninguna de las dos especies (Furné et al., 2012). Se ha observado que la inclusión de distintos niveles de carbohidratos en dietas de dorada provocó la disminución de ALT, mientras que la actividad de AST no se vio significativamente alterada respecto a control (Fernández et al., 2007). Furné et al. (2016) observaron que dietas ricas en lípidos y carbohidratos, con una menor relación de proteína/energía, aumentaban la actividad de ALT en trucha arcoíris, mientras que para *A. naccarii* estas dietas no afectaban a la actividad de ALT. En el mismo estudio, la actividad de AST no se vio afectada por los cambios dietarios en *A. naccarii*, a diferencia de lo observado en trucha arcoíris, donde su actividad aumentó al incrementar la proporción de lípidos y/o carbohidratos. GDH solo se vio afectada en dietas ricas en lípidos para *A. naccarii,* aumentando su actividad. También se han observado alteraciones en la actividad de estas enzimas relacionadas con el ciclo reproductivo, peso del animal y estación del año (Srivastava et al., 1999; Gómez-Milán et al., 2007; Jiang et al., 2015) en diferentes especies acuáticas. Las modificaciones en las fuentes lipídicas también modificaron la actividad de ALT y AST en *Oreochromis niloticus*, observándose una disminución en la actividad de estas enzimas al reemplazar total o parcialmente el aceite de pescado por otras fuentes lipídicas (Li et al., 2016a; Peng et al., 2016). Respecto a la sustitución de harina de pescado por fuentes alternativas de proteína, las modificaciones en la actividad de ALT y AST serán variadas y dependerán de la especie estudiada, su etapa de desarrollo, la fuente proteica por la cual se sustituye la harina de pescado, y el nivel proteico final de la dieta (Goméz-Requeni et al., 2004; Fournier et al., 2004; Cheng et al., 2010; Zhu et al., 2011; Li et al., 2014a; Kumar et al., 2017; Chaklader et al., 2020; Chemello et al., 2020; Mastoraki et al., 2020; Zhang et al., 2021; Cai et al., 2022).

1.3.4. Necesidades lipídicas

Los lípidos, además de ser una de las principales fuentes energéticas junto a las proteínas en peces, tienen otra serie de funciones: transportan compuestos liposolubles, actúan como precursores en la síntesis de hormonas, así como en compuestos funcionales como prostaglandinas y eicosanoides, participan en la estructura de membranas celulares y

subcelulares. Las necesidades lipídicas son difíciles de cuantificar debido a la complejidad composicional que presenta este tipo de compuestos. Los lípidos sirven principalmente como fuente energética, y los niveles de estos en dieta dependerá de los contenidos de proteína y carbohidratos. Una estrategia para minimizar la cantidad de proteína usada con fines energéticos es el "sparing" proteico. Este método consiste en aumentar la cantidad de lípidos en la dieta, para así destinar la mínima cantidad posible de proteína a fines energéticos, y por tanto aumentar el crecimiento (NRC, 2011). Los niveles de lípidos necesarios variarán de acuerdo con diversos factores, tales como la especie destinataria de la dieta, niveles y composición de la proteína y carbohidratos dietarios. La utilización de harina de pescado de diferente calidad proteica reveló que fueron necesarios mayores niveles de lípidos en la dieta con harina de pescado de menor calidad que en la dieta compuesta por harina de pescado de mejor calidad (Caballero et al., 1999; Vergara et al., 1999). La relación entre proteína y lípidos es otro factor relevante a la hora de determinar los niveles de lípidos necesarios en la dieta. Para dietas probadas en *Channa argus,* se observó que altos niveles de proteína, junto con 12-15 % de lípidos, tenían efectos negativos en el crecimiento, mientras que, en dietas con menor contenido proteico, dichos niveles de lípidos favorecían un mayor crecimiento que dietas con menores niveles de lípidos (Sagada et al., 2017). Resultados similares se han observado para *Pseudobagrus fulvidraco* y *O. niloticus* × *O. aureus*, para los que la inclusión de altos niveles de lípidos, junto con altos niveles de proteína, afectaba negativamente al crecimiento (Kim y Lee, 2005; Han et al., 2011). Por el contrario, las dietas para salmones pueden incorporar hasta un 35 % de lípidos (Rosenlund et al., 2016). De lo comentado, es fácil deducir que establecer un nivel adecuado de lípidos es difícil, aunque se debe incluir un mínimo de lípidos en la dieta que, además, cubra las necesidades de *n*-3 LCPUFA.

1.3.4.1. Ácidos grasos esenciales

La mayor parte de los lípidos ingeridos por los animales acuáticos están formados por ácidos grasos, por lo que las necesidades lipídicas especificas suelen expresarse como necesidades de ácidos grasos. Estas necesidades dependerán de las funciones que realizan y la capacidad del organismo de sintetizarlos endógenamente.

Los peces no pueden sintetizar PUFA *de novo*, por lo que tienen que obtener estos a través de la dieta. Los PUFA tienen un papel fundamental en todos los animales, ya que forman parte de los fosfolípidos que constituyen la membrana celular, les confieren propiedades

fisicoquímicas y participan en las funciones de las proteínas de la membrana (Tocher, 1998). En concreto, los peces requieren una mayor cantidad de *n*-3 PUFA a diferencia de los animales terrestres, los cuales presentan mayores necesidades de *n*-6 PUFA. Esto puede ser debido a que los *n*-3 PUFA permiten una mayor insaturación, lo que les confiere mayor flexibilidad y permeabilidad al formar parte de la membrana celular (Lovell, 1998). Los peces de agua dulce y diádromos también requieren *n*-6 PUFA, aunque la temperatura juega un papel relevante en las necesidades de *n*-3 y *n*-6 PUFA (NRC, 2011).

Entre los PUFA destacan acido linolénico (18:3*n*3, ALA) y ácido linoleico (18:2*n*6, LA), que sirven de precursores en la síntesis de los *n*-3 LCPUFA, EPA y DHA, y *n*-6 LCPUFA, ácido araquidónico (20:3*n*6, ARA), respectivamente (**Figura 14**). La mayoría de los peces marinos carecen de las enzimas necesarias para llevar a cabo la biosíntesis de EPA, DHA y ARA a partir de LA y ALA, por lo que necesitan la adición de estos en la dieta, principalmente EPA y DHA. Por otro lado, los peces de agua dulce presentan los mecanismos necesarios para biosintetizar EPA y DHA a partir de LA y ALA, por lo que sus necesidades se centran en el suministro dietario de estos últimos (Xie et al., 2021).

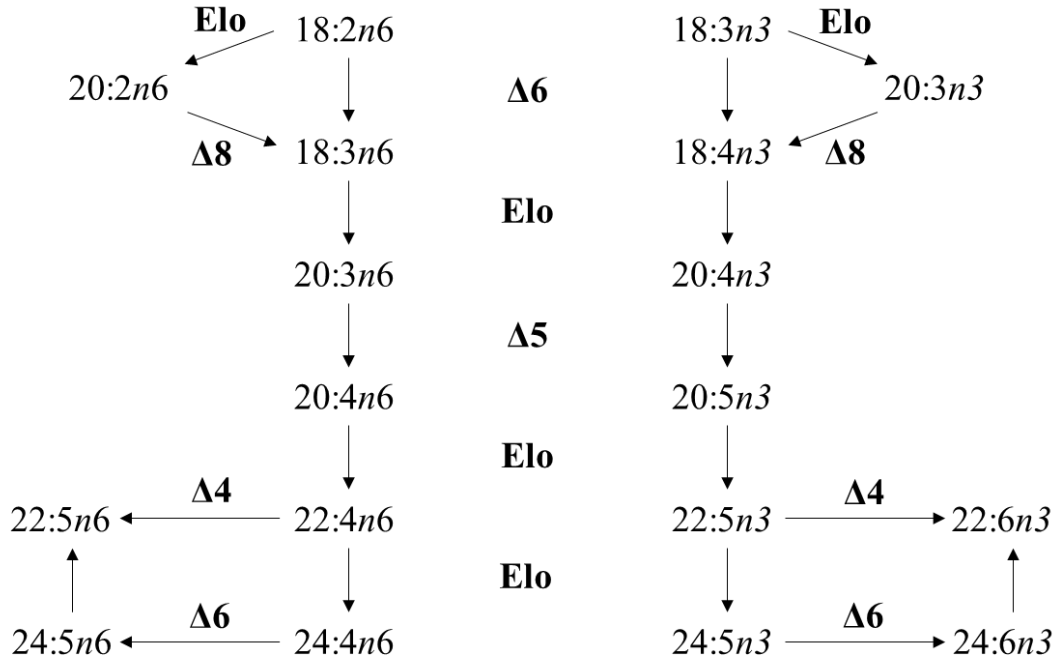


Figura 14. *Ruta metabólica para la síntesis de ácidos grasos. Δn hace referencia a distintas desaturasas y Elo hace referencia a elongasas.*

Para peces juveniles de agua dulce y diádromos, las necesidades de *n*-3 y *n*-6 PUFA varían según la especie y su hábitat, aunque por lo general las necesidades no suelen superar el 1 % del total de la dieta. En peces de aguas más frías, como *Salmo salar*, se recomienda la adición de *n*-3 LCPUFA en la dieta. En peces marinos, las recomendaciones de *n*-3 LCPUFA para larvas y alevines aumentan, y su rango se establece en 1-5,5 % en dieta. En juveniles, las recomendaciones disminuyen y se sitúan en 0,8-2,5 % de la dieta (NRC, 2011).

La sustitución de aceite de pescado por fuentes alternativas de lípidos con cantidades inferiores de EPA, DHA y ARA conlleva, por lo general, una disminución de estos ácidos grasos en el músculo del animal, incluso cuando se cubren sus necesidades (Nasopoulou y Zabetakis, 2012; Ti et al., 2019; Mastoraki et al., 2020). Está sustitución se debe realizar, tal y como se ha visto, teniendo en cuenta las necesidades de ácidos grasos esenciales y, a su vez, la capacidad de biosintetizar LCPUFA a partir de PUFA.

1.4. Microbiota intestinal en peces

Al igual que ocurre en todos los animales, el tracto digestivo de los peces se encuentra habitado por poblaciones de microorganismos. Estos microorganismos, según su procedencia, pueden ser alóctonos o autóctonos, aunque estos últimos pueden verse afectados en su estructura poblacional por la microbiota presente en el agua o el alimento (NRC, 2011). Dicha estructura poblacional, en cuanto a composición se refiere, muestra ciertas diferencias con la típica de los animales vertebrados terrestres. Así, los filos dominantes en las comunidades microbianas intestinales en peces son Proteobacteria, Firmicutes y Bacteroidetes (Ghanbari et al., 2015), recientemente renombrados como Pseudomonadota, Bacillota y Bacteroidota (Oren y Garrity, 2021). No obstante, y como es de esperar, la diversidad del microbioma intestinal se ve afectada por distintos factores, principalmente hábitat y dieta (Sullam et al., 2012; Egerton et al., 2018; Kim et al., 2021). El estudio realizado por Kim et al. (2021), basado en la microbiota intestinal de 227 peces de 85 especies diferentes, estableció Pseudomonadota, Bacillota, y Cyanobacteriota como filos dominantes entre los 21 detectados. Entre los tres filos acapararon más del 70 % de la población microbiana asociada al tracto intestinal, aunque la identificación a mayor nivel taxonómico se vio significativamente afectada por las características del hábitat en el que el pez se desarrolló y, en menor medida, por la naturaleza de su dieta. También se ha observado que la diversidad del microbioma aumenta al disminuir el nivel trófico de los organismos hospedadores (Sullam et al., 2012). Esto se ha confirmado en otros

experimentos en los que se comparaban las poblaciones microbianas en peces carnívoros, omnívoros y herbívoros (Larsen et al., 2014; Givens et al., 2015). Tal hecho puede ser debido a que las dietas omnívoras y herbívoras presentan una mayor variedad de sustratos para los microorganismos.

Como se ha comentado previamente, la dieta es uno de los factores que condiciona la composición y distribución de la microbiota intestinal. Se ha observado que variaciones de la dieta en acuicultura generan diferencias en el microbioma de los peces. Es el caso de la utilización de fuentes proteicas alternativas a la harina de pescado (Desai et al., 2012; Bruni et al., 2018; Huyben et al., 2019; Terova et al., 2019; Antonopoulou et al., 2019; Gaudioso et al., 2021; Reda et al., 2022). En concreto, la sustitución de harina de pescado por harina de insecto conlleva cambios en la microbiota de varias especies. En el estudio llevado a cabo por Antonopoulou et al. (2019), donde se utilizaron dietas en las que se sustituía parcialmente harina de pescado por harina de TM, y se alimentaron con ellas tres especies comerciales de acuicultura, los resultados mostraron cambios en la diversidad de la comunidad microbiona, principalmente en dorada y *Dicentrarchus labrax*, mientras que en trucha arcoíris los cambios fueron más leves. Huyben et al. (2019) analizó al microbiota de trucha arcoíris alimentado con dietas con inclusión parcial de harina elaborada con HI; los autores observaron un aumento en la diversidad y en bacterias del ácido láctico, así como en especies con capacidad de síntesis de enzimas lipolíticas y quitinolíticas. Esta última respuesta a la presencia de harina de HI en la dieta de trucha arcoíris ha sido también observada por otros autores (Gaudioso et al., 2021).

Además de los aspectos ambientales y nutricionales comentados, las características de la comunidad microbiana propia del tracto gastrointestinal de los peces presentan un componente genético. Así se deduce a partir de los resultados obtenidos por diferentes estudios, entre los que cabe citar el realizado por Roeselers et al. (2011). En este trabajo, llevado a cabo con *Danio rerio* cultivado en diferentes localizaciones, se realizaron comparaciones con individuos capturados en su hábitat natural. Los resultados mostraron que, a pesar de presentar claras diferencias en relación con sus hábitats, estos peces compartían un conjunto básico de poblaciones microbianas. Es decir, existía un número de especies siempre presentes, independientemente de las condiciones externas en las que el pez se desarrollase. Del mismo modo, en otros estudios se ha observado como la genética juega un papel importante en la diversidad del microbioma, al comparar diferentes especies criadas bajo mismas condiciones (Navarrete et al., 2012; Franchini et

al., 2014; Li et al., 2014b). Por tanto, y tomando en consideración todas las influencias e interacciones que afectan la estructura de la microbiota intestinal, se puede afirmar que, hasta cierto punto, las características y singularidades propias de cada animal modelan la composición de su microbioma (Roeselers et al., 2011).

La importancia de la comunidad microbiana intestinal es grande, ya que estos microorganismos participan en procesos fisiológicos, nutricionales, y de desarrollo del hospedador (Ye et al., 2014). Se ha demostrado que las bacterias colaboran en la producción de enzimas digestivas, lo que facilita la hidrolisis del alimento ingerido. Estas enzimas pueden digerir compuestos nutricionales que el hospedador no puede hidrolizar con enzimas endógenas (Ray et al., 2012). Por otro lado, las bacterias pueden llegar a sintetizar compuestos que facilitan el crecimiento y desarrollo del hospedador (Mountfort et al., 2002; Tsuchiya et al., 2008).

Otro factor relevante en acuicultura en relación con los microorganismos es el uso de antibióticos. Aunque cada vez más regulado, su aplicación a la hora de tratar infecciones o prevenirlas puede afectar negativamente a la población microbiana intestinal, ya que, al ser antibióticos de amplio espectro, no solo afectan al patógeno en cuestión, sino que atacan a especies propias del pez (Romero et al., 2014). Esto puede llevar a una disminución, tanto en número como en diversidad, de la microbiota del hospedador y, por tanto, a la reducción de la barrera protectora que supone la comunidad microbiana. En este sentido, el uso de compuestos prebióticos, tales como la quitina, puede favorecer la diversidad y estimular aumentos poblacionales de microorganismos con efectos beneficiosos para el organismo hospedador (Rimoldi et al., 2023).

1.5. Antibióticos en acuicultura

Debido al incremento de producción de la industria acuícola, el uso de antibióticos ha ido en aumento. Este uso no solo se limita al tratamiento de enfermedades infecciosas, dado que también se aplica para promover el crecimiento y reducir el riesgo de infección de forma preventiva. Los antibióticos actúan frente a bacterias a través de diferentes mecanismos, tales como inhibición de la síntesis proteica, inhibición de la síntesis de la pared celular, o inhibición de la síntesis de ácidos nucleicos (Kümmerer, 2009). Los antibióticos usados en acuicultura se pueden clasificar según su estructura en tetraciclinas, sulfonamidas, quinolonas, aminoglucósidos, macrólidos, fenicoles, β-lactamas, nitrofuranos, lincosamidas, y polimixinas. Los más usados son quinolonas (27 %), tetraciclinas (20 %), fenicoles (18 %), y sulfonamidas (14 %) (Schar et al., 2020). Muchos

de los antibióticos comúnmente usados en acuicultura también se aplican en el tratamiento de infecciones en humanos (Chen et al., 2020).

En el año 2017 se estimó que los antibióticos destinados a acuicultura alcanzaron 10.259 toneladas, y que está cifra crecería un 33 %, llegando a situarse en 13.600 toneladas en el año 2030 (Schar et al., 2020). La región que mayor porcentaje de aplicación acumula es Asia-Pacifico con 93,8 %, seguida de África y Europa. Se espera que para el año 2030, África y América del Sur sean regiones que aumenten la cantidad de antibióticos destinados a la industria acuícola, mientras que para la región de Asia-Pacifico y Europa se prevé estancamiento y disminución, respectivamente (Schar et al., 2020). Si comparamos el porcentaje de antibióticos que se destinan a acuicultura (5,7 %), frente a los destinados para la producción de animales terrestres (73,7 %), la cifra puede resultar pequeña. No obstante, el riesgo sanitario y ambiental que supone el uso de antibióticos en la industria acuícola no es despreciable. La forma más común de administrar antibióticos es oralmente a través del alimento, por lo que una vez procesado este, entre el 30-90 % de los antibióticos son excretados en heces u orina (Sapkota et al., 2008; Rico et al., 2013). De esta forma, se facilita que este tipo de compuestos o sus derivados metabólicos se difundan y acumulen en el medio acuático.

La acumulación y difusión de antibióticos puede generar efectos adversos en el ecosistema, tales como cambios en el microbioma del hábitat como consecuencia de la presión selectiva que ejercen, favoreciendo así el incremento poblacional de las especies resistentes al antibiótico, efectos tóxicos en otras especies presentes en el ecosistema, o impacto negativo en la diversidad y el desarrollo del zooplancton y fitoplancton (Buschmann et al., 2012; Samuelsen et al., 2014; Song et al., 2016; Okeke et al., 2022). Esto último puede ocasionar graves consecuencias en la cadena trófica del ecosistema. Además de los problemas asociados a la diversidad y desarrollo poblacional del ecosistema, el uso extendido de antibióticos afecta directamente a los seres humanos. El consumo de residuos de antibióticos presentes en productos acuícolas puede producir reacciones alérgicas o tener efectos tóxicos en nuestro organismo (Liu et al., 2017; Limbu et al., 2018; Okocha et al., 2018). Esto no solo se limita al consumidor, sino que afecta también a los trabajadores de la industria acuícola encargados de manipular los alimentos y antibióticos suministrados a los animales (Cabello, 2006). Otro aspecto importante, resultante del uso de antibióticos comunes a los usados en infecciones humanas, es la aparición de bacterias resistentes a antibióticos, que pueden transmitirse desde los

organismos acuáticos hasta humanos. Esto, además de incidir sobre la gravedad del proceso infeccioso, ocasiona efectos sobre el microbioma intestinal humano, ya que potencia la acción de patógenos resistentes en su interacción con las especies propias del tracto intestinal (Kruse y Sørum, 1994).

Debido a los problemas asociados al uso de antibióticos, muchos países, como los pertenecientes a la Unión Europea, han limitado el número de antibióticos permitidos en acuicultura y los casos en los cuales se pueden aplicar (Lulijwa et al., 2019; Pepi y Focardi, 2021). Además, la Unión Europea ha establecido niveles máximos de antibióticos presentes en productos acuícolas importados (Okocha et al., 2018). Esto abre el camino a la búsqueda de estrategias de control y prevención alternativas, más sostenibles y con menos implicaciones de carácter negativo derivadas de su aplicación (Wang et al., 2017; Mondal y Thomas, 2022; Okeke et al., 2022).

1.5.1. Polifenoles como agentes antimicrobianos

Los polifenoles son metabolitos secundarios que las plantas producen como respuesta al estrés. En la naturaleza se han descrito más de 10.000 polifenoles, que pueden clasificarse por su estructura en flavonoides y no flavonoides. Estos polifenoles surgen a partir de precursores comunes, como son la fenilalanina o el ácido siquímico. Estos compuestos pueden encontrarse en su forma libre o unidos a otros tipos de moléculas, tales como carbohidratos, lípidos, aminas, u otros polifenoles. La distribución de los polifenoles en los tejidos vegetales es variada; mientras los polifenoles hidrófobos se encuentran en la pared celular, los hidrófilos aparecen en las vacuolas de las células vegetales. Por lo general, las zonas más expuestas y, por lo tanto, más exteriores de las plantas, presentan una mayor concentración de polifenoles (Pandey y Rizvi, 2009). En este sentido, también influyen otro tipo de factores de carácter ambiental, así como el estado sanitario de la planta.

Los polifenoles tienen una amplia gama de actividades biológicas relacionadas con animales acuáticos, entre las que se encuentran actividades antibacterianas, antioxidantes, promotoras del crecimiento, antiinflamatorias, inmunoestimulantes y antihiperglucémicas (Taguri et al., 2006; Bouarab-Chibane et al., 2019; Tinh et al., 2021; Yang et al., 2021; Yuan et al., 2021; Ahmadi et al., 2022; Imperatore et al., 2023). Respecto a la actividad antibacteriana, se ha observado que los polifenoles presentan un rango variado de actuación, dependiendo del polifenol y la especie bacteriana (Taguri et al., 2006; Daglia, 2012; Bouarab-Chibane et al., 2019; Manso et al., 2021). Taguri et al.

(2006) observaron que los polifenoles extraídos y purificados de los tejidos vegetales presentaban una mayor actividad antibacteriana que los extractos vegetales compuestos por una mezcla de polifenoles y otras sustancias. También detectaron que, por lo general, los polifenoles que presentan en su estructura un sustituyente de pirogalol suelen exhibir una mayor actividad antibacteriana.

1.5.1.1. Polifenoles del té

Los polifenoles presentes en las hojas del té (*Camellia sinensi*) son principalmente flavonoides, de los cuales las catequinas representan el 80-90 % del total. Las catequinas agrupan a una familia de ocho compuestos denominados flavan-3-oles. Estas moléculas están compuestas por dos anillos de benceno y un heterociclo dihidropirano. Mediante la unión con moléculas de ácido gálico pueden formar conjugados esterificados. En la **Figura 15** se muestra la estructura de las principales catequinas presentes en el té.

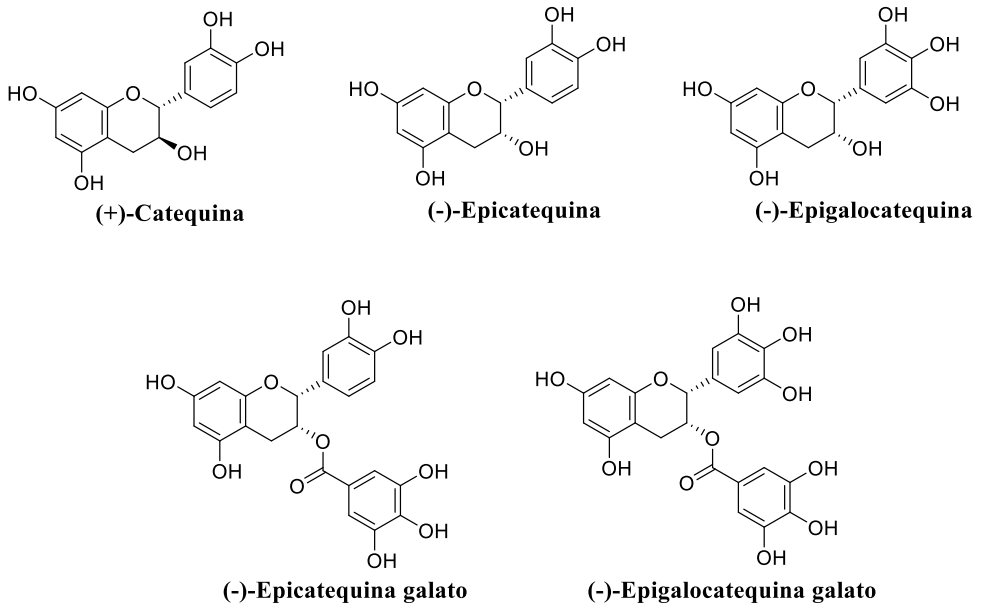


Figura 15. *Estructura de las cinco catequinas presentes en el té verde.*

Las catequinas presentan una alta actividad antioxidante, la cual pueden ejercer por mecanismo directo, atrapando especies reactivas de oxígeno o quelando iones metálicos, o indirecto, mediante interacción con el metabolismo antioxidante del organismo (Bernatoniene y Kopustinskiene, 2018).

La (-)-epigalatocatequina galato (EGCG) es la principal catequina en las hojas de té, representando entre el 50 y el 80 % del total de catequinas, además de ser la más activa, biológicamente hablando (Singh et al., 2011; Kim et al., 2014; Yan et al., 2020). En acuicultura, la suplementación de estos polifenoles de té en dietas se ha mostrado como una herramienta eficaz en cuanto a su incidencia sobre el crecimiento, actividad antibacteriana, efecto inmunoestimulante, antiviral, y antioxidante (Thawonsuwan et al., 2010; Wang et al., 2017; Ji et al., 2018; Zhang et al., 2020; Qian et al., 2021; Li et al., 2022; Zhang et al., 2022). La actividad antibacteriana de las catequinas y, en concreto, de la EGCG, se ha evaluado en diferentes especies bacterianas. Se ha observado que estas moléculas muestran actividad antibacteriana por sí mismas, aunque también presentan resultados positivos al combinarse con antibióticos, reduciéndose la cantidad mínima necesaria de estos últimos (Yanagawa et al., 2003; Taguri et al., 2006; Gordon y Wareham, 2010; Betts et al., 2011; Miklasińska-Majdanik et al., 2018; Betts et al., 2019; Álvarez-Martínez et al., 2021). Y lo que es más interesantes, la sinergia que pueden presentar las catequinas con antibióticos puede restaurar el poder antibacteriano de un antibiótico frente a cepas de bacterias con resistencia (Betts et al., 2019).

Sin embargo, los polifenoles del té presentan desventajas, siendo las más destacada la relacionada con su baja estabilidad en medios biológicos. Estos compuestos se degradan fácilmente a temperatura fisiológica, en presencia de oxígeno, frente a iones metálicos, y pH alcalino y neutro (Krupkova et al., 2016; Xu et al., 2019; Jin et al., 2022). Por lo tanto, la administración oral de polifenoles del té conlleva una baja absorción y una vida media corta (Dang et al., 2013). Para superar estos inconvenientes, la nanoencapsulación puede resultar una estrategia adecuada a la hora de administrar estas moléculas (Dang et al., 2015; Rambaran, 2020; Di Santo et al., 2021).

1.5.2. Nanoencapsulación de compuestos bioactivos

La nanotecnología tiene diferentes aplicaciones en la acuicultura, como la administración de moléculas bioactivas y vacunas, eliminación de contaminantes presentes en agua, detección de patógenos, actividad antimicrobiana y antiviral, y conservación del producto destinado a la venta (Shah y Mraz, 2019; Fajardo et al., 2022). La nanotecnología se ha aplicado en relación con la problemática derivada del uso de antibióticos en acuicultura. Algunas de las estrategias desarrolladas con este objetivo implican el uso de nanomateriales con actividad antibacteriana, degradación y eliminación de antibióticos

presentes en agua, sensores de presencia bacteriana, o el transporte de compuestos bioactivos con actividad antibacteriana.

Respecto a la nanoencapsulación de compuestos bioactivos, los biopolímeros han cobrado relevancia debido a su baja toxicidad y biodegradabilidad (Esfanjani y Jafari, 2016). La zeína es una proteína hidrofóbica que se encuentra en el maíz; su baja solubilidad en agua permite la formación de nanopartículas coloidales en agua (Pascoli et al., 2018), y se ha utilizado ampliamente para la encapsulación de compuestos activos tanto hidrofílicos como hidrofóbicos (Zhang et al., 2014; Nunes et al., 2020; Jin et al., 2022; Zheng et al., 2022). Sin embargo, las dispersiones de nanopartículas de zeína muestran una baja estabilidad coloidal, tendencia a la agregación y precipitación a pH 5-7 (Yuan et al., 2022). El recubrimiento de nanopartículas de zeína con otros biopolímeros mejora su estabilidad, y la presencia de diferentes grupos funcionales facilita la interacción entre las nanopartículas y las moléculas bioactivas, aumentando así la encapsulación (Yuan et al., 2022). En este aspecto, se han utilizado un gran número de compuestos para la estabilización de nanopartículas de zeína, como proteínas, lípidos, carbohidratos, etc. (Dai et al., 2016; Sun et al., 2020; Jiang et al., 2021; Zhou et al., 2021; Wang et al., 2023).

En la sección 1.2.8.3. se habló de la quitina presente en los insectos. Este polisacárido no digerible forma parte de la cutícula de los insectos y representa hasta un 36,6 % del total del peso en seco del insecto. La quitina se extrae de los insectos, generalmente por un proceso secuencial de desmineralización, desproteinización y blanqueado. La quitina, como polímero, presenta propiedades interesantes y existe un gran número de estudios que avalan su aplicación en diversas áreas (Khoushab y Yamabhai, 2010). Tal y como se dijo anteriormente, la quitina está formada por dos monómeros, *N*-acetilglucosamina y *N*-glucosamina, aunque es mayor el número de monómeros de *N*-acetilglucosamina en relación con el de *N*-glucosamina. Cuando se invierte la proporción, el polímero resultante se denomina quitosano. La relación entre el número de monómeros se puede expresar como grado de acetilación. El quitosano es un polisacárido natural y el único polímero catiónico presente en la naturaleza. Esta última característica propicia su solubilidad en medio ácido, a diferencia de la quitina. Este polímero ha atraído la atención en los últimos años debido a su biocompatibilidad, biodegradabilidad y baja toxicidad (Kozma et al., 2022). Sus áreas de aplicación, al igual que las de la quitina, son extensas, y abarcan la industria alimentaria, la industria farmacéutica, agricultura, acuicultura,

medicina, cosmética, etc. (Morin-Crini et al., 2019). El método más usado para la obtención de quitosano a partir de la quitina es a través de una hidrolisis alcalina con hidróxido de sodio (**Figura 16**) (Kozma et al., 2022).

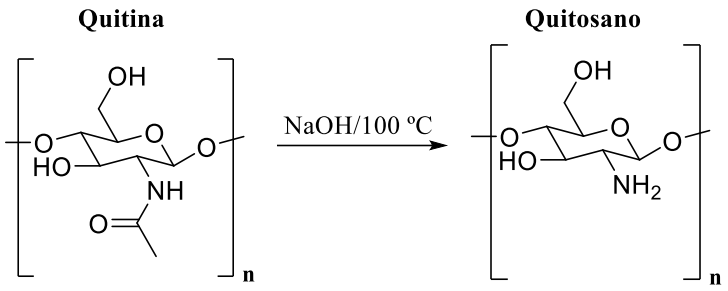


Figura 16. *Obtención de quitosano a partir de quitina.*

La estabilización de las nanopartículas de zeína con quitosano se ha evaluado en varios estudios con diferentes moléculas bioactivas encapsuladas (Park et al., 2015; Baspinar et al., 2018; Khan et al., 2019; Pauluk et al 2019; Zhou et al., 2021). El recubrimiento de las nanopartículas de zeína con quitosano se lleva a cabo mediante la técnica del recubrimiento capa por capa. Esta técnica se basa en la deposición electrostática; las nanopartículas se mezclan con una disolución de un material con carga opuesta, el material se unirá a la superficie de las nanopartículas de zeína (Yuan et al., 2021). Mediante esta técnica se pueden depositar varias capas de materiales que modifiquen las propiedades fisicoquímicas de las nanopartículas (Khan et al., 2019; Carrasco-Sandoval et al., 2021).

OBJETIVOS

El objetivo principal de esta tesis es la valoración del uso de insectos en acuicultura. Este objetivo principal se desglosó en distintos objetivos parciales:

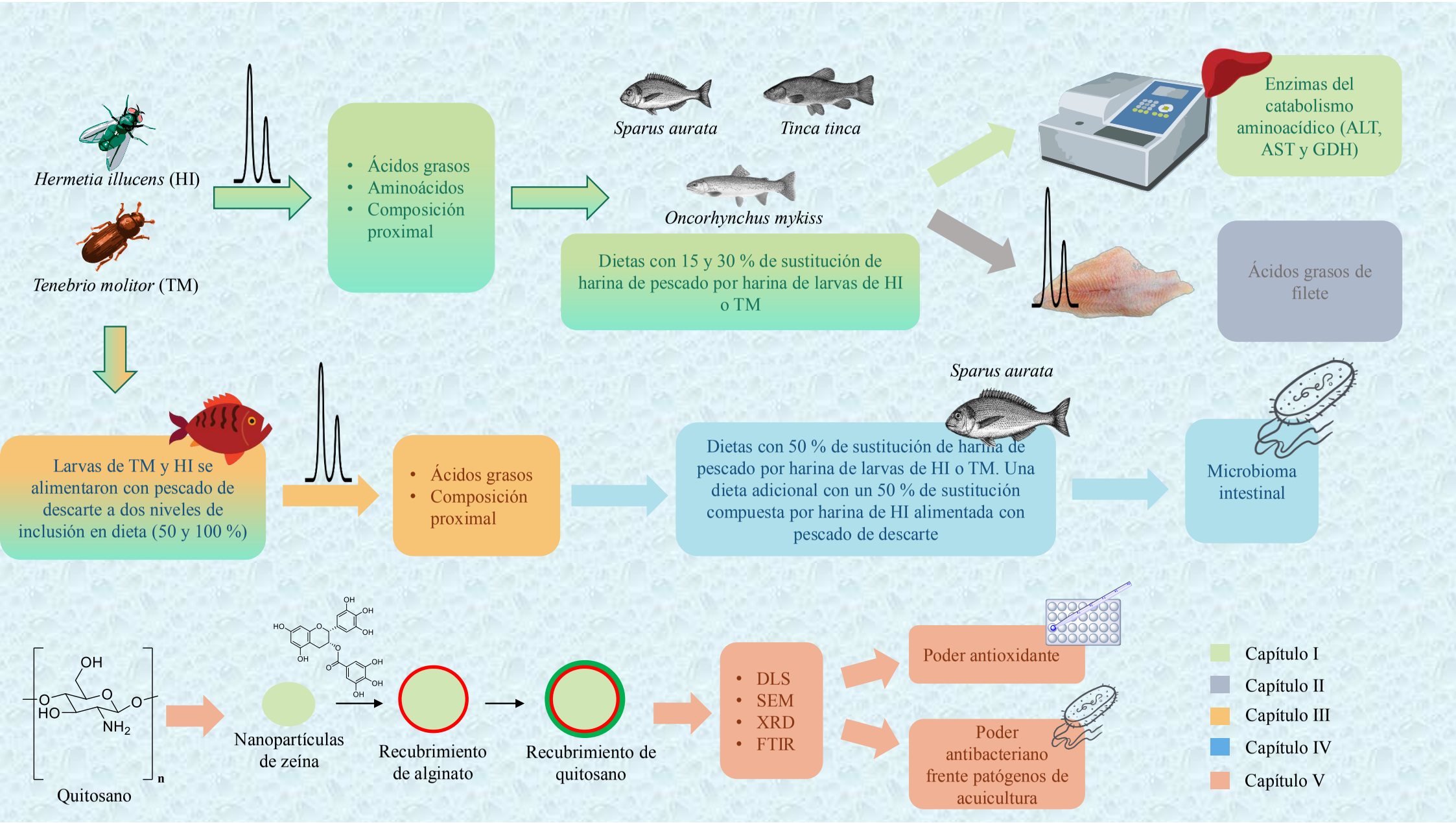
- Caracterización del perfil nutricional del insecto y su optimización mediante la alimentación.
- Respuesta de la inclusión de la harina de insecto en los parámetros fisiológicos y productivos de distintas especies de peces relevantes en la producción acuícola.
- Aprovechamiento de las fracciones indigeribles de los insectos en nanotecnología y su aplicación con fines higiénico-sanitarios.

OBJECTIVES

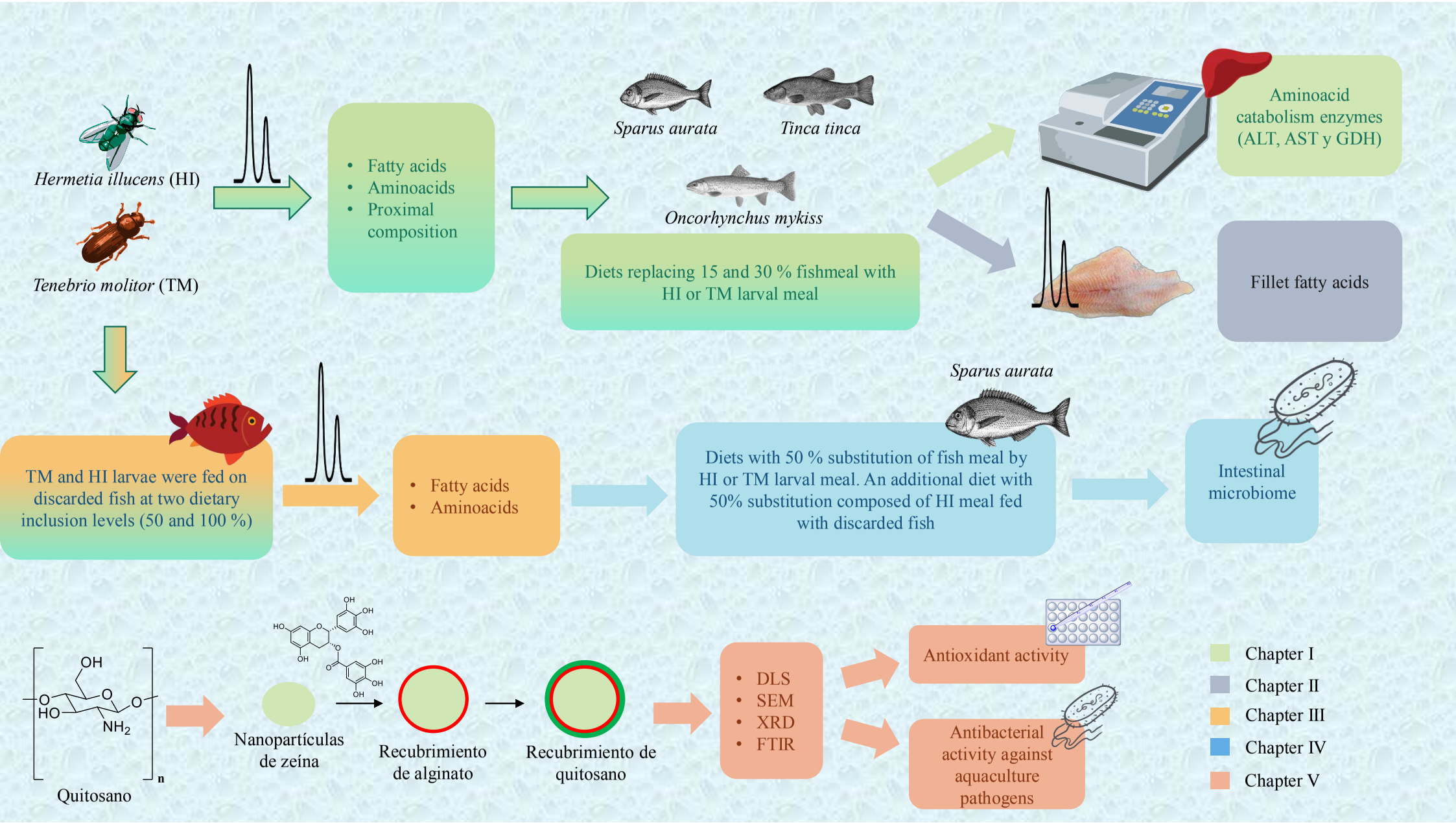
The main objective of this thesis is the assessment of the use of insects in aquaculture. This main objective was broken down into different sub-objectives:

- Characterization of the nutritional profile of the insect and its optimization through feeding.
- Response of the inclusion of insect meal on the physiological and productive parameters of different fish species relevant to aquaculture production.
- Exploitation of the indigestible fractions of insects in nanotechnology and their application for hygienic-sanitary purposes.

ESQUEMA EXPERIMENTAL


Hermetia illucens (HI)
Tenebrio molitor (TM)
• Ácidos grasos
• Aminoácidos
• Composición proximal
Sparus aurata
Tinca tinca
Oncorhynchus mykiss
Dietas con 15 y 30 % de sustitución de harina de pescado por harina de larvas de HI o TM
Enzimas del catabolismo aminoacídico (ALT, AST y GDH)
Ácidos grasos de filete
Larvas de TM y HI se alimentaron con pescado de descarte a dos niveles de inclusión en dieta (50 y 100 %)
• Ácidos grasos
• Composición proximal
Sparus aurata
Dietas con 50 % de sustitución de harina de pescado por harina de larvas de HI o TM. Una dieta adicional con un 50 % de sustitución compuesta por harina de HI alimentada con pescado de descarte
Microbioma intestinal
Quitosano
Nanopartículas de zeína
Recubrimiento de alginato
Recubrimiento de quitosano
• DLS
• SEM
• XRD
• FTIR
Poder antioxidante
Poder antibacteriano frente patógenos de acuicultura
Capítulo I
Capítulo II
Capítulo III
Capítulo IV
Capítulo V

EXPERIMENTAL SCHEME


Hermetia illucens (HI)
Tenebrio molitor (TM)
• Fatty acids
• Aminoacids
• Proximal composition
Sparus aurata
Tinca tinca
Oncorhynchus mykiss
Diets replacing 15 and 30 % fishmeal with HI or TM larval meal
Aminoacid catabolism enzymes (ALT, AST y GDH)
Fillet fatty acids
TM and HI larvae were fed on discarded fish at two dietary inclusion levels (50 and 100 %)
• Fatty acids
• Aminoacids
Sparus aurata
Diets with 50 % substitution of fish meal by HI or TM larval meal. An additional diet with 50% substitution composed of HI meal fed with discarded fish
Intestinal microbiome
Quitosano
Nanopartículas de zeína
Recubrimiento de alginato
Recubrimiento de quitosano
• DLS
• SEM
• XRD
• FTIR
Antioxidant activity
Antibacterial activity against aquaculture pathogens
Chapter I
Chapter II
Chapter III
Chapter IV
Chapter V

CAPÍTULOS EXPERIMENTALES

CAPÍTULO I: *Comparative study of growth performance and amino acid catabolism in* Oncorhynchus mykiss, Tinca tinca *and* Sparus aurata *and the catabolic changes in response to insect meal inclusion in the diet*

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Comparative study of growth performance and amino acid catabolism in *Oncorhynchus mykiss*, *Tinca tinca* and *Sparus aurata* and the catabolic changes in response to insect meal inclusion in the diet

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A B S T R A C T

This experiment studied the kinetics of three key enzymes involved in the hepatic amino acid metabolism, alanine aminotransferase (ALT, EC 2.6.1.2.) aspartate aminotransferase (AST, EC 2.6.1.1.) and glutamate dehydrogenase (GDH, EC 1.4.1.3.), of three fish species: rainbow trout (*Oncorhynchus mykiss*), tench (*Tinca tinca*) and gilthead seabream (*Sparus aurata*) and their response to two levels (15% and 30%) of fish meal replacement with two different insect meals (*Hermetia illucens* and *Tenebrio molitor*). Five iso- nutritional and isoenergetic diets were used; a control diet and four experimental diets by replacing fishmeal with insect meal from *H. illucens* or *T. molitor* at 15% and 30%. The daily intake was recorded, and the feeding trial lasted 46 days for trout, 43 days for seabream and 100 days for tench. Initial weight for each fish species was 55.40 g for trout, 17.75 g tench and 6.79 g for seabream.

The results showed different percentages of weight gain among species, being 293% for Seabream, followed by trout, 155%. The lowest weight gain was found in tench, 78%, without statistic differences for weight gain among treatment except for seabream feed with H-30 showed the lowest percentage of weight gain. Regarding enzymes activity, the results showed different kinetic parameters for the three fish species according their nutritional needs. A high Vmax, Km and catalytic efficiency for ALT were observed in tench; however, for AST, the highest Vmax was observed in seabream and tench, which also showed the highest catalytic efficiency. Minimal differences in GDH kinetics were found among species; the Vmax in trout was slightly lower than it was in tench.

The inclusion of insects affected the different fish species in different ways. In trout, the inclusion of insect meal tended to increase the Vmax of the three enzymes and the Km and catalytic efficiency of GDH. In seabream, the inclusion of insect meal increased the Vmax of ALT and increased the Km of AST However, the catalytic efficiency of GDH decreased with the inclusion of insects, particularly the *T. molitor* meal. Finally, the tench did not show significant differences with the inclusion of insect meal except a strong interaction for H-30, which could be due an imbalance in essential amino acids/no essential amino acids. Regarding the effect of insect species, in trout AST Km increased with *H. illucens* meal while in gilthead seabream increased when *T. molitor* were included. These different metabolic adaptations seem to be consequence of the nutritional habit of each specie in addition with the amino acids bioavailability of each diet.

1. Introduction

In recent years, the interest in insects as an alternative protein source has increased. Insects are being evaluated as a food for humans (Patel et al., 2019, Barroso et al., 2017.) and as a protein source for animals (Sánchez-Muros et al., 2014; Van Huis et al., 2013). In this context, the use of insect meal instead of fishmeal as a protein source in aquaculture has been widely studied (Henry et al., 2015).

Fishmeal is indispensable in aquaculture; however, it is a finite resource that generates environmental problems, which make it neither economically nor environmentally sustainable (Sánchez-Muros et al., 2014). Insect meal is posited as a very interesting protein source. From an environmental point of view, insect cultures are more sustainable; culturing insects does not require large areas or large quantities of water, and it contributes to the recycling of by-products and waste, which reduces the carbon footprint (Blonk et al., 2008 I). The species that have attracted the most interest due to their nutritive value, the possibility of mass rearing and the ability to control their life cycle are the yellow mealworm (*T. molitor*) (Iaconisi et al., 2017; Secci et al., 2018), the common house fly (*Musca domestica*) (Allegratti et al., 2018; Hussein et al., 2017) and the black soldier fly (*H. illucens*) (Schiavone et al., 2017, 2018).

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The inclusion of *T. molitor* meal has been studied in several fish species such as African catfish (*Clarias gariepinus*) (Ng et al., 2001), European seabass (*Dicentrarchus labrax*) (Gasco et al., 2016; Gasco et al., 2014), blackspot seabream (*Pagellus bogaraveo*) (Iaconisi et al., 2017), rainbow trout (*Oncorhynchus mykiss*) (Belforti et al., 2015), yellow catfish (*Pelteobagrus fulvidraco*) (Su et al., 2017), tilapia (*Oreochromis niloticus*) (Sánchez-Muros et al., 2016) and gilthead seabream (*Sparus aurata*) (Piccolo et al., 2017). In general, major growth was observed around 25% of replacement of fish meal depending of fish species. The highest percentage of replacement, 75%, were obtained for yellow catfish (Su et al., 2017) without compromising the growth, nevertheless the quality of fillet was altered, especially fatty acid profile, by the *T. molitor* meal inclusion (Iaconsi et al., 2017; Sánchez-Muros et al., 2016)

H. illucens meal has been tested in European seabass (Magalhães et al., 2017), rainbow trout (Renna et al., 2017; Sealey et al., 2011; Stadtlander et al., 2017), Pacific white shrimp (*Litopenaeus vannamei*) (Cummins et al., 2017), *Ictalurus punctatus*, blue tilapia (*Oreochromis aureus*) (Bondari and Sheppard, 1987), Jian carp (*Cyprinus carpio* var. Jian) (Li et al., 2017), yellow catfish (Xiao et al., 2018) and Nile tilapia (*Oreochromis niloticus*) (Devic et al., 2018). The results for *H. illucens* are similar to those obtained for *T. molitor*, allowing a percentage of fish meal substitution around of 25% without compromising the growth, nevertheless changes in fatty acid profile of fillet were observed. (St-Hilaire et al., 2007). When defatted meal were used or insect meal enriched in n-3 long chain fatty acids, the percentage of inclusion increase until 50% without negative effect on growth (Renna et al., 2017; Stadlander et al., 2017), or fatty acid composition of fillet (Sealey et al., 2011).

These experiments have focused mainly on growth, nutritive parameters, digestive efficiency and the effect on the quality of the final product; the effect on amino acid metabolism has not been studied.

The utilization of protein for growth purposes depends on the nutritional characteristics of the feed and the protein source, but it is necessary to emphasize the physiological characteristics of the species. Fish species differ in their nutritional habits, nutritional needs and the assimilation efficiency of dietary protein. It is therefore necessary to accommodate the particular protein needs of different species to improve the efficiency and assimilation of amino acids. The catabolizing enzymes of amino acids are considered a good indicator of amino acid utilization (Fournier et al., 2003; Gómez- Requeni et al., 2003; Lupiánez et al., 1989; Peres and Oliva-Teles, 2005; Sánchez-Muros et al., 1998).

In this experiment, three different enzymes directly related to amino acid metabolism have been analysed. Alanine aminotransferase (ALT, EC 2.6.1.2.) and aspartate aminotransferase (AST, EC 2.6.1.1.) are the enzymes that catalyse the transamination reactions of alanine and glutamic and aspartic acid. Glutamate dehydrogenase (GDH, EC 1.4.1.3.) plays an important role in fish metabolism due to its catalysis of the deamination of L-glutamate (Fig. 1).

The amino acids and protein needs depend on the feeding habit of the species, in consequence the amino acids metabolism as well the adaptation to changes in diet will vary over the specie. Carnivorous species, trout and gilthead seabream, have a high protein requirement. Rainbow trout and gilthead seabream are considered carnivorous species with a crude protein requirement of 43% (FAO, 2019a) and 45–50% (FAO, 2019b) for juveniles, respectively. The feeding habits of tench are not completely known. According to Michel and Oberdorff (1995) tench can be considered carnivorous and they feeding aquatic micro invertebrates. On the opposite, Benzer et al. (2009) have been described tench as omnivorous fish. The protein requirement for tench has been established greater than 35% for adults (de Pedro et al., 2001) and 48–52% for juveniles (González-Rodríguez, 2015).

The kinetics of these three key enzymes of hepatic amino acid metabolism were studied in three fish species: rainbow trout (*O. mykiss*), tench (*T. tinca*) and gilthead seabream (*S. aurata)* together with their metabolic response to the partial replacement of fish meal with two different insect meals (*H. illucens* and *T. molitor*).

2. Materials and methods

2.1. Insect rearing and diet formulation

H. illucens and *T. molitor* were purchased from Entomotech S.L. and MealFood Europe S.L., respectively, and then dried and ground into insect meal, Table 1 shows the gross composition and amino acidic profile of insects used in this experiment. The formulation of diets was prepared in Technical services of University of Almeria (Spain). Five isonutritional experimental diets were prepared: one control diet, two diets with 15% of fishmeal replaced by *H. illucens* meal or *T. molitor* meal (H-15 and T15, respectively) and two diets with 30% of fishmeal replaced by insect meal (H-30 and T30, respectively). Fish meal (Guadalen S.A., Murcia-Spain) used had 12% of lipids and 69% of crude protein. Table 2 shows the proximal composition and ingredients of the insect meal and the formulated diets. The gross composition of diets used in this experiment was analysed according to the standard procedures of the Association of Official Analytical Chemists (AOAC, 2005).

2.2. Fish feeding trials

The fish used for this experiment were juveniles of the three different species: *S. aurata, O. mykiss* and *T. tinca*. For each species, 450 fish were used for the experiment after an adaptation period of 15 days to the aquarium conditions. The fish were weighed and randomly distributed among five groups (C, H-15, H-30, T-15 and T-30) with three tanks (300*L*) per group. In the case of gilthead seabream, the fish were supplied by Predomar S.L. (Carboneras, Almería) and were held under constant temperature conditions (20 ± 1 °C) and a natural photoperiod in facilities at Almería University. The rainbow trout were acquired from a commercial farm (Piscifactoría Fuente del Campillo, Guadalajara, Spain) and were cultured at the facilities of ITACyL at 15 ± 1 °C with a photoperiod 12 h light:12 h dark. The juvenile tench were maintained in facilities at the Aquaculture Centre of Vegas del Guadiana (from Junta de Extremadura). Both temperature (22.4 ± 1.4 °C) and photoperiod (14 L:10 D) were held constant.

The fish were hand fed ad libitum twice a day at 09:00 and 14:00. The daily intake was recorded, and the feeding trial were lasted the weight 46 days for trout, 40 days seabream. Due to the low growth rate of tench the trial was prolonged until 100 days. The initial weights are recorded in Table 3.

2.3. Sampling and liver extract preparation

At the end of the experiment, the fish were fasted for 24 h before sampling. The fish from all groups were anaesthetized, and weighed individually. Six fish per tank were sacrificed by administering an overdose of anaesthesia (clove oil 100 mg/L), and then the liver were immediately frozen in liquid N_2 and stored at −80 °C for subsequent analysis.

To analyse apparent digestibility, faeces were collected during the last two weeks of the growth trial by a modified Guelph method (Cho et al., 1982), gathering the faeces produced throughout 24 h in a settling column. The faeces samples were frozen at −20 °C until their analysis.

The liver was homogenized (1:9, *w/v*) with a buffer solution (Tris/HCl 0.1 M, Triton X-100 0.1%, EDTA 0.25 mM and NaCl 0.01 M pH 7.6) using a Polytron PT 2100 (Kinematica AG Inc., Lucerne, Switzerland) in an ice bath. The homogenate was centrifuged at 15,000 rpm for 15 min at 4 °C (Orto Alresa Biocen 22r). The resulting homogenates were stored at −80 °C until enzyme analysis.

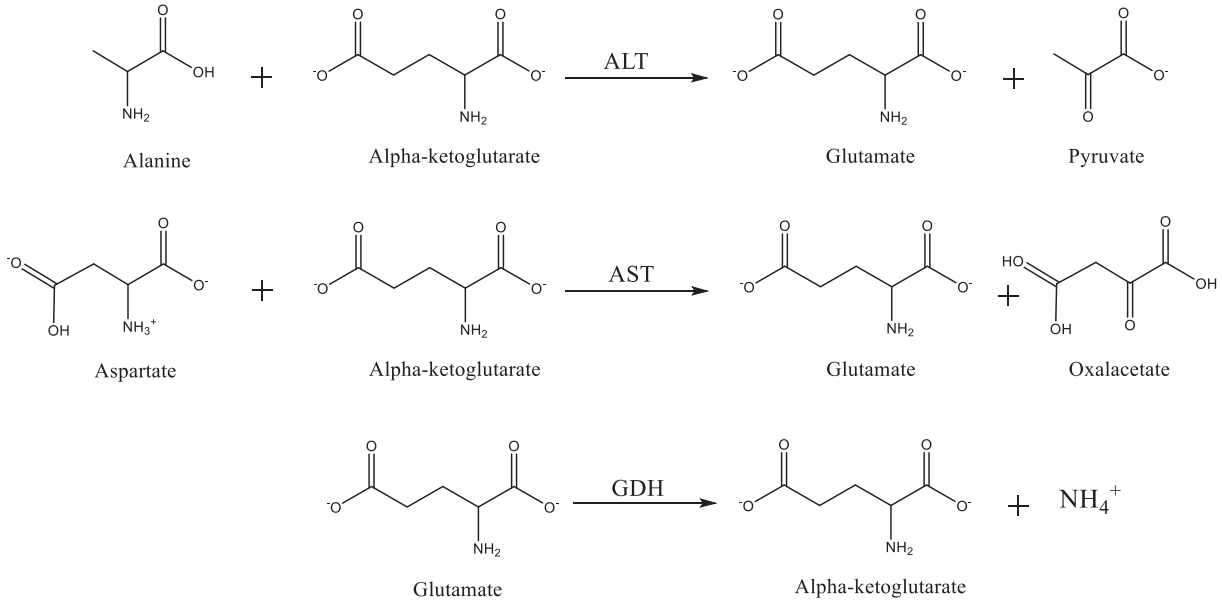

Fig. 1. Enzymatic catalysis carried out by ALT, AST and GDH.

Table 1
Gross composition and amino acids of insect meals from *Tenebrio molitor* and *Hermetia illucens* used in the experiment.

	Hermetia illucens	*Tenebrio molitor*
Proximate composition (g/100 g dry matter)		
Crude protein	40.1	55.3
Crude fat	33.9	28.3
Ash	10.6	3.7
Chitin	16.6	5.9
Amino acid composition (g/100 g dry matter)		
Asp (Aspartate)	2.68	3.34
Thr (Threonine)	0.77	1.24
Ser (Serine)	1.21	2.08
Glu (Glutamate)	2.83	4.82
Gly (Glycine)	1.58	2.32
Ala (Alanine)	2.27	3.40
Cys (Cysteine)	0.08	0.11
Val (Valine)	1.22	1.87
Met (Methionine)	0.40	0.51
Ile (Isoleucine)	0.80	1.22
Leu (Leucine)	1.66	2.53
Tyr (Tyrosine)	1.41	2.39
Phe (Phenylalanine)	1.08	1.53
His (Histidine)	0.66	1.32
Lys (Lysine)	1.38	2.13
Arg (Arginine)	1.05	1.93

Table 2
Ingredients and proximate experimental diets composition.

	Diets				
	C	H15	H30	T15	T30
Ingredients (g/100 g dry matter*)*					
Fishmeal	36.8	31.5	25.9	31.3	25.9
Hermetia illucens	0.0	5.6	10.9	0.0	0.0
Tenebrio molitor	0.0	0.0	0.0	5.1	10.7
Wheat gluten	10.5	12.7	14.8	12.1	12.6
Soy protein concentrate	15.1	15.9	17.0	15.1	16.5
Wheat meal	16.2	14.0	11.9	15.6	14.6
Soy lecithin	1.3	1.0	1.0	1.0	0.8
Fish oil	11.7	10.8	9.8	11.3	10.7
Vitamins and minerals	2.0	2.0	2.0	2.0	2.0
Goma guar	2.0	2.0	2.0	2.0	2.0
Hemoglobin	3.9	3.9	3.9	3.9	3.9
Methionine	0.08	0.15	0.22	0.15	0.22
Lysine	0.56	0.58	0.60	0.59	0.61
Total	100	100	100	100	100
Proximate composition (% dry matter*)*					
Crude protein	45.8	46.6	45.4	46.5	46.3
Crude fat	16.5	17.4	17.3	17.4	16.8
Crude fiber	1.5	1.9	1.5	1.3	1.9
Ash	8.1	7.9	7.8	7.5	8.2

Vitamin and mineral premix (mg/kg): vitamin A 2000,000 UI; vitamin D3: 200,000 UI; vitamin E: 12,000; vitamin K3: 2600; vitamin B1: 3000; vitamin B2: 3000; vitamin B6: 2000; vitamin B9:1500; vitamin B12: 10; vitamin H: 300; inositol: 50,000; betaine: 50,000; calcium pantothenate: 10,000; nicotic acid: 20,000; Co: 60; Cu: 900; Fe: 600; I: 50; Mn: 950; Se: 1; Zn: 750; Ca: 190,000; K: 24,000; Na: 41,000.

2.4. Enzyme kinetic assays

Alanine aminotransferase was measured using the method described by Lain-Guelbenzu et al. (1991) with different concentrations of L-alanine (0.01, 0.1, 1, 10, 25 and 50 mM) as a substrate. Aspartate aminotransferase was measured using the method described by Krista and Fonda (1973) with different concentrations of L-aspartate (0.01, 0.1, 1, 10, 25 and 50 mM) as a substrate. Glutamate dehydrogenase was measured using the method described by Katoh et al. (2003) with different concentrations of α-ketoglutarate (0.01, 0.05, 0.1, 0.5, 1 and 10 mM) as a substrate. The reactions catalysed by these enzymes are shown in Fig. 2.

The determination of enzyme activity was based on the spectrophotometric measurement of the decrease in optical density caused by the oxidation of nicotinamide adenine dinucleotide (NADH). The ideal protein concentration for each extract was determined in a previous test that quantified the protein in the extracts using a Pierce protein assay kit (Thermo-Fisher). The reactions were launched by the addition of the substrate to the combined assay mixture and enzyme extract. The oxidation of NADH was measured at 340 nm in UV/vis mode using a Power Wavex microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) in duplicate in 96-well microplates (UV-Star Greiner Bio-One, Frickenhausen, Germany).

The activity of all enzymes was expressed as milliunits of activity per milligram of protein. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol of NADH per min at 25 °C.

Table 3
Effect on weight gain and feed utilization of different treatment at the end of the feeding trial.

	Control	H-15	H-30	T-15	T-30
Gilthead seabream					
Wi	6.79 ± 0.36	6.74 ± 0.02	6.78 ± 0.02	6.74 ± 0.02	6.79 ± 0.01
Wf	26.44 ± 0.38	25.94 ± 0.83	23.70 ± 1.01	25.33 ± 0.15	25.97 ± 0.31
WG (%)	293.0 ± 5.33 ^{b}	284.4 ± 12.02 ^{ab}	249.4 ± 13.58 ^{a}	275.7 ± 3.58 ^{ab}	282.2 ± 3.89 ^{ab}
FCR	1.02 ± 0.00	0.98 ± 0.01	0.92 ± 0.12	1.01 ± 0.01	1.03 ± 0.01
Tench					
Wi	17.75 ± 0.32	18.70 ± 0.39	18.65 ± 0.40	17.94 ± 0.21	18.19 ± 0.23
Wf	31.60 ± 0.22	31.90 ± 1.52	33.58 ± 0.77	33.15 ± 0.36	32.4 ± 0.65
WG (%)	78.07 ± 2.07	70.37 ± 4.59	80.02 ± 0.89	84.80 ± 2.67	78.17 ± 3.24
FCR	1.82 ± 0.04	1.90 ± 0.08	1.77 ± 0.04	1.60 ± 0.02	1.89 ± 0.12
Rainbow trout					
Wi	55.44 ± 0.86	54.88 ± 0.30	56.46 ± 0.70	53.70 ± 0.43	55.98 ± 0.75
Wf	141.5 ± 1.20	139.5 ± 1.65	141.0 ± 0.71	140.0 ± 0.81	141.0 ± 0.81
WG (%)	155.3 ± 2.24	154.2 ± 3.16	149.9 ± 1.88	160.9 ± 0.85	151.2 ± 2.21
FCR	0.768 ± 0.00 ^{ab}	0.778 ± 0.01 ^{b}	0.782 ± 0.01 ^{b}	0.754 ± 0.00 ^{a}	0.773 ± 0.01 ^{ab}

Means (± SE) of three replicate tanks (30 fish/tank). Different letter indicates significant differences ($p < .05$) based on Tukey-Kramer HSD test. Wi: initial weight; Wf: final weight; Weight gain (WG): (Wf – Wi/Wi) x 100; Feed conversion ratio (FCR): feed intake/weight gain (g). Survival in all diets and species were 100%.

2.5. Kinetic parameters

All kinetic parameters were obtained with Statgraphics 18 by subjecting the data to the non-linear regression methods described by the Michaelis-Menten equation: V = Vmax([S]/ (Km + [S])), where V the initial velocity of the reaction; [S] is the substrate concentration; Vmax the maximum initial velocity, which is related to the "saturation" of the enzyme by an infinite concentration of substrate; and Km is the Michaelis-Menten constant, which is numerically equivalent to the concentration of the substrate at half-maximal initial velocity. Catalytic efficiency (CE) measures the efficiency of converting substrate to product by an enzyme, i.e., Vmax/Km.

2.6. Estimate digestibility of insect meal

The digestibility of CP of insect meals under study were estimated according to the equation proposed by Sugiura et al., 1998.

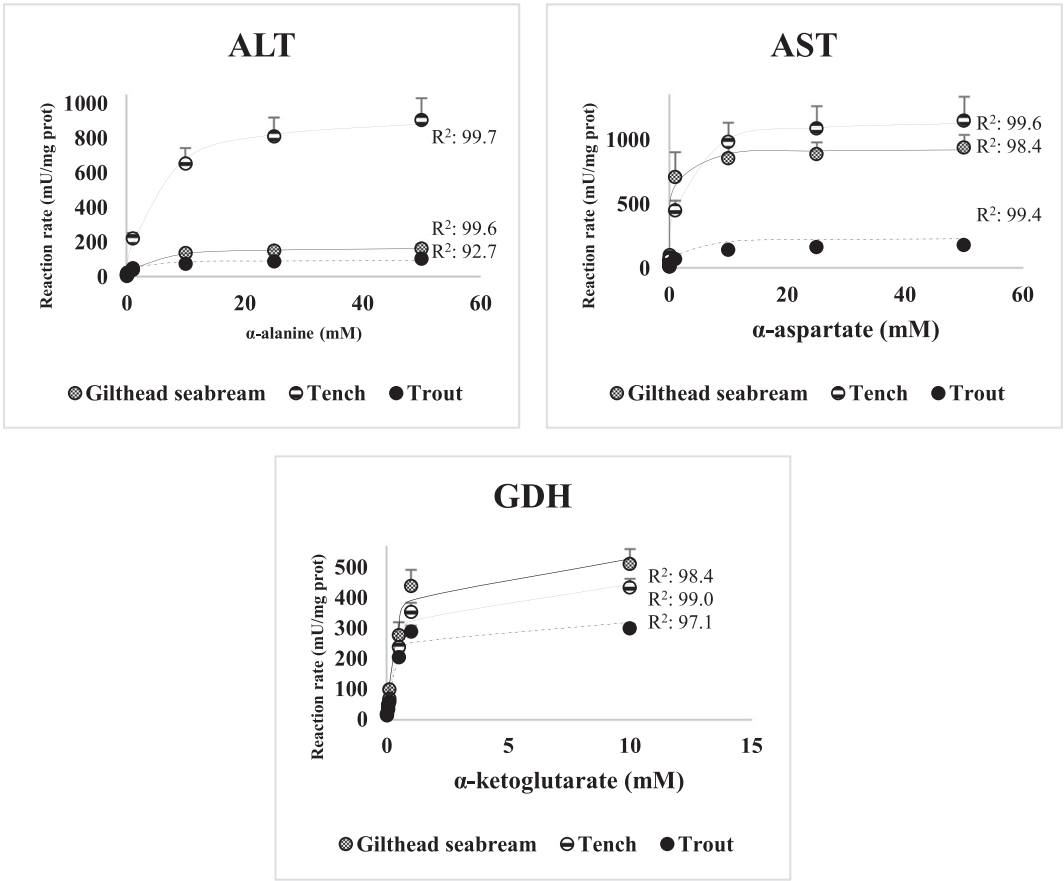

Fig. 2. Kinetic curves of three ALT, AST and GDH for Gilthead seabream, tench and trout fed with control diet. Specific activity, are expressed as mU/mg protein. Values are means ± S.E.M. $n = 6$. R^2: adjustment to Michaelis-Menten equation.

$$ADi\% = ADCt\left[\left(\frac{(1-S)Db}{sDT}\right)X\,(ADCt - ADCb)\right]$$

where ADi is the apparent digestibility of insect meal under study (%), ADCt is the apparent digestibility coefficient of the experimental diet, ADCb is the apparent digestibility coefficient of the control diet (%), Db is the protein of the basal diet (%), Dt is the protein of the test experimental diet (%), s is the proportion of the insect meal in experimental diet and 1-s is the proportion of the fish meal diet in the experimental diet.

Digestibility of diet were determined by acids using acid in soluble ash as marker in feeds and faeces (Atkinson et al., 1984).

2.7. Statistical analysis

The statistical software used was IBM SPSS Statistics 25. The results were analysed using a one-way analysis of variance (ANOVA) followed by the Tukey-Kramer HSD test. The results are expressed as the mean and SEM (standard error of mean) with probabilities of $P < .05$ considered significant. Kinetic parameters for ALT, AST and GDH (Table 5) were analysed by two-way analysis of variance (ANOVA) (species and diet).

The fit to Michaelis-Menten curve has been made with Statgraphics Centurion 18.

2.8. Ethical standards

The experiment was conducted according to the directive 2010/63/EU of the European Parliament for the protection of animals used in experiments and for other scientific purposes and was approved by the Órgano Competente of Junta de Andalucia (n° ref.: RTA2015–00021-CO3–02) according to Royal Decree 53/2013 of 1 February 2013.

3. Results

3.1. Growth and nutritive indices

As shown in Table 3, no statistic differences were found for weight gain among treatment in tench and rainbow trout while seabream the fish feed with H-30 showed the lowest percentage of weight gain. Regarding diet utilization only statistic differences between T-15 and trout fed with *H. illucens* meal.

Nevertheless, the percentage of weight gain varies among species, being 293% for Seabream, followed by trout, 155%. The lowest weight gain was found in tench, 78% of initial weight and 100 days, while the trial of trout and seabream were lasted 46 and 40 days respectively.

Regarding estimated digestibility (Table 4) of insect meal in general trout and tench show higher digestibility for insect meal than seabream. No statistic differences were found in trout among treatments. In tench only T-15 showed less digestibility regarding the other treatments while in seabream *H. illucens* diet showed less digestibility at two levels of inclusion than *T. molitor* diets, showing higher digestibility at 30% of inclusion.

3.2. Comparisons among species

Fig. 2 shows the saturation curves of ALT, AST and GDH for the species studied when fed with the control diet. In all cases, the enzymatic activity showed Michaelis-Menten behaviour.

Regarding the kinetic parameters (Table 5), the lowest values of Vmax for ALT were observed in rainbow trout (94.8 mU/mg prot) followed by gilthead seabream (172.8 mU/mg prot); the highest (and statistically significant) Vmax was observed for tench (1066 mU/mg prot). The Km values differed among species, being highest in tench (4.1 mM) followed by gilthead seabream (2.42 mM) and trout (0.8 mM). The lowest catalytic efficiency was found in gilthead seabream (64.7), which was followed by trout (133.1) and tench (265). No significant difference was found in Km for AST, although for Vmax the lowest value was observed in trout (237.9 mU/mg prot), which was followed by gilthead seabream (1037.1 mU/mg prot) and tench (1297.7 mU/mg prot). Similar to ALT, the catalytic efficiency of AST was 8–10 times higher for tench (825) than for trout (174.8) and gilthead seabream (82.9). The Km for GDH was not significantly different among the species; however, the Vmax was higher in gilthead seabream (552.1 mU/mg prot) than it was in trout (332.8 mU/mg prot), whereas tench (471 mU/mg prot) was intermediate. For this enzyme, the catalytic efficiency was similar for the three species.

The results (F-values) of two-way analysis of variance (Table 6) show a strong interaction ($p < .0001$), for Vmax, Km and CE for the three enzymes measured except Km of GDH that (p-value $< .05$).

3.3. Effects of insect inclusion

3.3.1. Alanine transaminase

The inclusion of insect meal in the diet had an impact on amino acid metabolism depending on the fish species and the diet. Table 5 displays the ALT data obtained for the three fish species studied fed with diets containing two different levels of substituted insect meals.

In trout, the Vmax of the enzyme increased, and was significantly different for fish fed with the H-15 and T-30 diets compared with fish fed the control diet. For gilthead seabream, the inclusion of insect meal increased Vmax with significant differences for the H-15, T-15 and T-30 diets compared with the control diet. Tench showed a statistical increase in Vmax for the H-30 diet compared with the other three insect-based diets.

The Km in the three fish was not significantly different among the diets. The catalytic efficiency in trout and tench was not significantly different between insect meal diets and the control diet. In gilthead seabream, the CE increased for all insect meal diets and was significantly different from the control diet; the highest CE was observed for the H-15 diet.

3.3.2. Aspartate transaminase

The enzyme AST showed different behaviours among the different fish species (Table 5). In trout, the inclusion of insects increased the Vmax regardless of the insect species or the inclusion level while tench only showed statistic differences between H-30 and T-30, for gilthead seabream, the Vmax of AST did not change in fish fed insect-based diets. The Km values were significantly different only in the gilthead seabream, which showed an increased Km that in fish fed with the and T-30 diets significantly different to Km of H-15 and control.

The CE showed a pattern similar to Vmax for trout, increasing with the inclusion of insect meal and being significantly different compared

Table 4
Estimated percentage of apparent digestibility of CP of insect meals for the four experimental diets with insects.

	H-15	H-30	T-15	T-30
Gilthead (%)	82.15 ± 4.63 ^{b}	71.57 ± 0.47 ^{a}	89.06 ± 0.93 ^{c}	101.5 ± 0.27 ^{d}
Tench (%)	96.47 ± 1.70 ^{b}	96.84 ± 0.47 ^{b}	91.18 ± 2.00 ^{a}	96.09 ± 0.33 ^{b}
Trout (%)	100.7 ± 10.64	94.44 ± 6.92	96.36 ± 7.19	98.90 ± 0.66

Means (± SE) of three replicate tanks. Different letter indicates significant differences ($p < .05$) based on Tukey-Kramer HSD test.

Table 5
Effect of insect meal inclusion on kinetic parameters of liver enzymes (ALT, AST, GDH) for the three fish species feed with the experimental diets.

		Control	H-15	H-30	T-15	T-30
Alanine aminotransferase (ALT)						
Gilthead	Vmax	172.8 ± 23.1 ^{aA}	317.7 ± 37.1 ^{b}	271.6 ± 16.9 ^{ab}	373.7 ± 35.8 ^{b}	300.3 ± 14.3 ^{b}
	Km	2.4 ± 0.3^{B}	1.6 ± 0.4	1.9 ± 0.2	2.3 ± 0.1	2.1 ± 0.2
	CE	64.7 ± 3.1 ^{aA}	216.8 ± 25.5 ^{c}	142.6 ± 7.7 ^{b}	191.2 ± 11.5 ^{b}	147.1 ± 9.8 ^{b}
Tench	Vmax	1066.0 ± 83.0 ^{abB}	868.4 ± 45.3 ^{a}	1366.5 ± 118.5 ^{b}	898.2 ± 55.7 ^{a}	835.1 ± 90.4 ^{a}
	Km	4.1 ± 0.3	3.7 ± 0.2	3.8 ± 0.4	3.5 ± 0.2	4.0 ± 0.4
	CE	265.0 ± 25.2 ^{abC}	236.0 ± 18.0 ^{a}	411.4 ± 57.7 ^{b}	270.3 ± 33.0 ^{ab}	215.9 ± 15.7 ^{a}
Trout	Vmax	94.8 ± 12.2 ^{aA}	202.2 ± 22.6 ^{c}	120.7 ± 13.7 ^{ab}	142.0 ± 11.0 ^{abc}	155.6 ± 9.2 ^{bc}
	Km	0.9 ± 0.2^{A}	0.9 ± 0.2	1.3 ± 0.2	1.4 ± 0.2	0.6 ± 0.1
	CE	133.2 ± 19.4 ^{abB}	234.9 ± 35.2 ^{b}	84.2 ± 5.2 ^{a}	97.8 ± 18.4 ^{a}	233.8 ± 35.0 ^{b}
Aspartate aminotransferase (AST)						
Gilthead	Vmax	1037.1 ± 93.3 ^{B}	954.9 ± 111.9	759.0 ± 103.6	912.5 ± 91.3	752.9 ± 49.9
	Km	1.1 ± 0.1 ^{ab}	1.3 ± 0.1 ^{abc}	0.8 ± 0.1 ^{a}	1.7 ± 0.3 ^{bc}	1.9 ± 0.1 ^{c}
	CE	987.1 ± 215.4 ^{bA}	733.6 ± 69.4 ^{ab}	940.9 ± 111.5 ^{b}	779.9 ± 151.3 ^{ab}	390.9 ± 19.0 ^{a}
Tench	Vmax	1297.7 ± 179.1 ^{abB}	1287.3 ± 147.4 ^{ab}	1585.3 ± 101.5 ^{b}	960.9 ± 153.4 ^{ab}	820.8 ± 136.3 ^{a}
	Km	1.2 ± 0.2	1.3 ± 0.1	1.1 ± 0.1	1.1 ± 0.0	1.1 ± 0.1
	CE	824.8 ± 123.3 ^{abB}	1190.4 ± 89.6 ^{b}	1358.5 ± 97.0 ^{b}	908.7 ± 154.2 ^{ab}	629.3 ± 152.9 ^{a}
Trout	Vmax	238.0 ± 22.5 ^{aA}	551.3 ± 30.8 ^{b}	610.3 ± 40.9 ^{b}	613.6 ± 32.4 ^{b}	613.4 ± 12.3 ^{b}
	Km	1.5 ± 0.3	2.5 ± 0.3	2.3 ± 0.3	1.5 ± 0.2	1.7 ± 0.2
	CE	173.8 ± 26.7 ^{aA}	220.8 ± 21.7 ^{ab}	255.1 ± 24.4 ^{abc}	399.6 ± 64.5 ^{c}	376.5 ± 32.2 ^{bc}
Glutamate dehydrogenase (GDH)						
Gilthead	Vmax	552.0 ± 54.9 ^{dB}	448.5 ± 48.1 ^{bd}	254.1 ± 15.7 ^{ac}	388.3 ± 23.2 ^{bc}	230.5 ± 23.1 ^{a}
	Km	0.4 ± 0.1	0.5 ± 0.0	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
	CE	1393.0 ± 88.1 ^{c}	1002.1 ± 163.7 ^{bc}	718.1 ± 79.6 ^{ab}	637.0 ± 56.5 ^{ab}	405.6 ± 51.5 ^{a}
Tench	Vmax	471.0 ± 37.6 ^{aAB}	506.2 ± 29.6 ^{a}	885.1 ± 33.9 ^{b}	676.3 ± 88.2 ^{ab}	628.1 ± 43.4 ^{a}
	Km	0.5 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.1
	CE	1194.2 ± 229.6 ^{a}	1373.2 ± 144.3 ^{a}	3248.0 ± 170.9 ^{b}	1508.8 ± 116.1 ^{a}	1895.4 ± 364.6 ^{a}
Trout	Vmax	331.8 ± 22.9 ^{aA}	1425.4 ± 88.6 ^{c}	980.0 ± 85.4 ^{b}	1049.9 ± 77.0 ^{b}	1673.2 ± 79.0 ^{c}
	Km	0.3 ± 0.1 ^{a}	0.6 ± 0.1 ^{b}	0.4 ± 0.0 ^{ab}	0.5 ± 0.1 ^{ab}	0.5 ± 0.0 ^{ab}
	CE	1141.0 ± 208.5 ^{a}	2468.0 ± 200.1 ^{bc}	2266.9 ± 211.2 ^{b}	2269.4 ± 268.7 ^{b}	3220.7 ± 95.2 ^{c}

Means (± SE) from 6 individual measurements. Different minuscule superscript letters indicate statistical difference ($p < .05$) within the different treatment and different capital superscript letters indicate statistical difference ($p < .05$) within the same kinetic parameter of different fish species fed with control diet based on Tukey-Kramer HSD test. Km, Vmax and catalytic efficiency (CE) are expressed as mM. $mU{\cdot}mg^{-1}$ of protein and $U{\cdot}mM^{-1}{\cdot}mg^{-1}$ of protein respectively.

Table 6
Results (F-values) of two-way analysis of variance for the data in Tables .

	Specie	Diet	Interaction
Alanine aminotransferase (ALT)			
Vmax	456.6 ^{c}	5.1 ^{b}	9.2 ^{c}
Km	171.1 ^{c}	n.s.	n.s.
CE	38.0 ^{c}	3.5 ^{a}	9.1 ^{c}
Aspartate aminotransferase (AST)			
Vmax	53.0 ^{c}	2.8 ^{a}	4.9 ^{c}
Km	19.2 ^{c}	n.s.	4.4 ^{b}
CE	50.1 ^{c}	4.7 ^{b}	3.7 ^{b}
Glutamate dehydrogenase (GDH)			
Vmax	199.6 ^{c}	18.4 ^{c}	34.9 ^{c}
Km	4.4 ^{a}	3.3 ^{a}	n.s.
CE	98.3 ^{c}	10.7 ^{c}	18.3 ^{c}

Significance levels are ^{a}, $p < .05$; ^{b}, $p < .005$; ^{c}, $p < .0001$; n.s., not significant ($p > .05$).

with the control for both the T-15 and T-30 diets. For gilthead seabream, the CE decreased in fish fed insect meal and was statistically lower for fish fed the T-30 diet compared with both the control diet and H-30 diets. In tench, the CE was not significantly different for insect meal diets compared with the control diet, increase of CE were observed in two *H. illucens* based diets regarding T-30 diet.

3.3.3. Glutamate dehydrogenase

The kinetic parameters of GDH, Vmax and Km (Table 5) also showed different responses depending on the fish species. In trout, the inclusion of insect meal increased the Vmax in fish fed insect meal diets, particularly the H-15 and T-30 diets. In contrast, in gilthead seabream, the Vmax decreased significantly for both the H-30 and T-30 diets. In tench, there was a slight increase in activity with the inclusion of insect meal, but this was only statistically significant in fish fed the H-30 diet compared with the control diet.

Regarding the Km, no changes were observed in gilthead seabream or tench, whereas in trout, the Km tended to decrease in fish fed insect meal, but this was only significantly different between the H-15 diet and the control diet. No differences were observed among the other treatments.

In gilthead seabream, the CE tended to decrease in fish fed insect meal diets; this represented a statistically significant difference between the H-30, T-15 and T-30 diets relative to the control. For tench, only fish fed the H-30 diet showed a significant difference relative to the other four diets. In trout, however, the CE tended to increase in fish fed insect meal and was significantly different for all fish-based diets compared with the control diet being the higher value for T-30.

3.3.4. Interaction between species and diet

The two-way ANOVA (Table 6) showed a dependence of kinetic parameter and diet except for Km of ALT and AST with an interaction between diet and species for Vmax and CE of three enzymes measured while for Km this interaction only appears for AST enzyme.

4. Discussion

This experiment studied the kinetics of three of the most representative enzymes of amino acid metabolism—ALT, AST and GDH—as indicators of amino acid utilization (Fournier et al., 2003; Gómez-Requeni et al., 2003; Peres and Oliva-Teles, 2005, Sánchez-Muros et al.1998) in three different fish species fed two insect species meals as a replacement for fish meal.

4.1. Growth and diet utilization

The control diet shows different percentage of weight gain among the three fish species, being gilthead seabream and trout the species with major weight gain while tench show a.

low growth rate as described for this species (Erguden and Goksu, 2011; Webster and Jana, 2003;). The greater weight gains by gilthead seabream regarding trout could be due to the develop stage, even with the same age trout had an initial weight of 55.44 g whereas gilthead seabream weighed 6.74.

The response to insect inclusion varied among species, although in none of the three species there are differences in the final weight among diets, seabream tends to decrease the percentage of weight gain with insect inclusion, especially at highest levels of substitution with *H. illucens*. On the other hand, in rainbow trout insect meal inclusion affects the FCR, displaying better diet utilization for T-15 than other insect based feed. In tench the inclusion of insects had no effect on weight gain or diet utilization. These results showed that trout and tench, both freshwater fish, the inclusion of insect did not affect the weight gain or improved FCR while in gilthead seabream, a saltwater fish, tends to decrease the % weight regarding control diet especially at high levels of *H. illucens* inclusion.

These results could be related with the estimated digestibility of the source (Table 4) lower for seabream except in fish fed with T-30 diet. The lower digestibility of insect meal it is associated with chitin content, it is commonly assumed that fish cannot digest chitin (Rust, 2002) and may also prevent the absorption of proteins and lipids by the intestine (Tanaka et al., 1997). Nevertheless, chitinase and the other enzymes related with chitin hydrolysis have been evidenced in both freshwater (Jeuniaux, 1993; Lindsay et al., 1984;) and marine fish (Clark et al., 1988; Danulat and Kausch, 1984; Fänge et al., 1979; Fines and Holt, 2010; Kono et al., 1987; Kurokawa et al., 2004). Both freshwater and saltwater fish eat chitin in their natural diet, freshwater chitin come from insect and crustacean while the saltwater chitin come mainly from crustacea. The chitin of crustaceans is enclosed in a matrix of proteins (Johnson and Peniston, 1982; No et al., 1989), whereas chitin of insect is part of a matrix of proteins, lipids and other compounds (Kramer et al., 1995) which could be related with the estimated digestibility obtained in tench, being insects part of natural diet of tench and trout but not of seabream.

4.2. Comparisons among species

In this experiment, we determined the saturation curve of the three enzymes and calculated the kinetic parameters Km, Vmax and EC. The results showed a Michaelis-Menten adjustment for the three enzymes in the three fish species studied; nevertheless, the kinetic parameters Km and Vmax varied among species (Fig. 2 and Table 5).

The kinetic parameters determined showed a strong dependence of the species probably adapted to their nutritive habits.

For trout and gilthead seabream, ALT showed a low Vmax, which indicates a low level of enzymatic protein compared with the high Vmax observed in tench. However, the Km values were low in trout than in the other two species studied, which indicates that the ALT of trout is more effective at low substrate concentrations than tench, while gilthead seabream showed an intermediate affinity for the substrate. These data suggest a different metabolic response in these three fish species.

Carnivorous fish such as trout have been described as having poor molecular regulation of hepatic metabolism due to a lack of regulation of endogenous glucose production through gluconeogenesis (Marandel et al., 2015; Panserat et al., 2000, 2001) using dietary amino acids for endogenous glucose synthesis (Cowey et al., 1977; Moon and Foster, 1995) The enzyme ALT catalyses the transamination of amine groups to form pyruvate, a very common gluconeogenic substrate that is directly related to liver gluconeogenesis, a low Km values increase the degradation of amino acids at physiological concentration which guarantee a source for gluconeogenic substrate when the diet have not an excess of protein,.

In tench, ALT is more active at high substrate concentrations. The high Vmax observed in this fish could be related to an excess of dietary protein (Lupiánez et al., 1989) in accordance with the omnivorous habit described for this species (Benzer et al., 2009), and it probably requires less protein than established by Gónzalez-Rodriguez (2015), 48–52% for juveniles, and included in the diet formulation of this experiment (45.8% CP).

The results of AST activity in tench are consistent with those obtained for ALT and support the suggestion of excess dietary protein due to an omnivorous feeding habit. Attention is drawn to the high Vmax of gilthead seabream ALT relative to trout, although they are both carnivores. This situation was confirmed by the EC, which was higher for gilthead seabream than trout, indicating more transamination of amino acids by the AST pathway than the ALT pathway in gilthead seabream, whereas in trout the main pathway for catabolism of amino acids uses ALT. These different way to utilize the amino acids indicates different nutritional needs, trout obtained pyruvate, through ALT, to glucose production through the gluconeogenesis pathway, whereas gilthead seabream obtain oxaloacetate, through AST, to use as energy through TCA path.

Despite the differences in transaminase activity, GDH activity (Table 5) did not show substantial differences among the fish species studied, and the species also exhibited a similar EC. These results are in contrast to the high transaminase activity found in tench, which should be reflected in a high GDH activity. Actually, it is difficult to explain, since GDH deaminated the glutamate obtained in transamination reactions. Dean et al. (1986) found GDH activity decreased, GOT activity increased, and GPT activity remained unchanged, as dietary protein quality was raised. The high activity of GDH implies high nitrogen production which have harmful effects on fish (Wright, 1995). To alleviate these negative effects, the ammonium can be fixed on glutamate to form glutamine through glutamine synthetase (Ip and Chew, 2010).

In gilthead seabream and trout GDH activity is in accordance with transaminases activities, the highest values of Vmax of gilthead seabream could be related to the high Vmax in AST.

These data are consistent with those regarding glucose utilization; in rainbow trout the administration of glucose or ingestion of a high carbohydrate diet results in poor dietary glucose utilization and a prolonged hyperglycaemia (Bergot, 1979; Brauge et al., 1995; Palmer and Ryman, 1972). Omnivorous species such as the common carp or tench use more efficiently high levels of dietary carbohydrates. Gilthead seabream has been described as an intermediary phenotype (Furuichi and Yone, 1981, 1982a, 1982b; Wilson, 1994) between trout and tench.

4.3. Inclusion of insects

The replacement of fishmeal with insect meal led to changes in amino acid catabolism in the fish species studied. The data show a strong interaction between diet and fish species for Vmax and CE of three enzymes determined, nevertheless for Km do not show this interaction in GDH with similar values among treatment and fish species.

In trout, the inclusion of insects tends to increase amino acid catabolism. Both ALT and AST increased their activity when insect protein was increased, but no change in the affinity of the enzyme for the substrate was observed. Nevertheless, the CE only increased for AST with the inclusion of insect meal, particularly with the inclusion of *T. molitor*, whereas ALT showed a CE similar to the control that indicate an increase of amino acids utilization for energetic purpose when *T. molitor* are included. The enzyme GDH responded to the inclusion of insect meal with increased activity of the enzyme at the saturated substrate concentration via an increase in the quantity of the enzymatic protein and a decreased affinity for the substrate, which translates into an increase in EC. These results indicate a major utilization of amino acids

for gluconeogenic and energetic purposes when insects are included in the diet. This nutritional situation is compatible with a high protein diet (Cowey et al., 1977; Sánchez-Muros et al., 1998) or an intake of low quality of protein (low EAA/NEAA) (Peres and Oliva-Teles, 2006) in carnivorous fish as have been described (Bañuelos-Vargas et al., 2014; Fournier et al., 2003; Gómez-Requeni et al., 2003; Moyano et al., 1991; Peres and Oliva-Teles, 2005, 2006; Peres and Oliva-Teles, 2007).

In gilthead seabream, the replacement of fishmeal with insect meal also tended to increase ALT activity via an increase in the synthesis of enzymatic proteins, whereas AST tended to decrease activity only at subsaturated substrate concentration via conformational changes in the enzyme.. The inhibition of AST at a subsaturating substrate concentration allow to keep the physiological concentrations of amino acids catabolized trough AST while the high Vmax allows to catabolize the excess of amino acids to obtain energy. This variation on Km and Vmax provoke an increase of ALT CE and a decreased AST CE. As result of this some balance could be created in amino acids catabolism that tend to beak with the greatest level of insects inclusion (T-30 and H-30) decreasing Vmax and CE of GDH. These results indicate that the lower percentage of weight gain in gilthead seabream fed with H-30 are more related with the low digestibility of the *H. illucens* meal, and in consequence could increase the misbalance of EEAA/NEAA, and the amino acid used to gluconeogenic or energetic purposes, while the percentage of weight gain for T-30 seem to indicate that the low activity of GDH are related with major synthesis of protein for growth. This differences between *H. illucens* and *T. molitor* meal could be due to a major percentage of amino acids and digestibility of this source for gilthead seabream. Piccolo et al. (2017) also obtain an increase of digestibility al 25% of replacement with *T. molitor* meal in gilthead seabream. No data of *H. illucens* digestibility for gilthead seabream were found but low growth rates obtained by Karapanagiotidis et al. (2014) at 30% of replacement, would be explained by the low digestibility and they would agree with the data obtained in this experiment.

In tench, the major effect on insect meal was an increase in the activities of the three measured enzymes in the H-30 treatment, which reveal a strong interaction between fish species and diet. The lack of an effect on enzyme activities in other three treatments could be related to the level of protein inclusion in the diet, which could mask the effect of insect meal. Nevertheless, the high activity observed in the H-30 diet is difficult to explain. One explanation could be the different essential amino acid content in *T. molitor* meal compared with *H. illucens* meal. *T. molitor* meal showed a higher percentage of essential amino acids than *H. illucens* meal, which at high levels (30%) could provoke a decrease in the EAA/NEAA. Dietary AA imbalances have also been reported to affect specific activities of AA deamination and transamination enzymes (Fournier et al., 2003; Gómez-Requeni et al., 2003). The low GDH activity could be related with the glutamate and glutamine increase in liver, and with the reduction of accumulation of essential free amino acids in muscle and reduction of hepatic and circulating alanine described by Roques et al. (2020) in trout fed with hydrolysate of insect protein. On the other hand, the natural habitat of tench are areas with low water flow, shallow areas of lakes and ponds with a preference for muddy or sandy substrates (Rendon et al., 2003) also it has great resistance to low concentrations of dissolved oxygen in water and a certain degree of eutrophication and is well adapted to wide range of pH and temperature (Lukowicz and Proske, 1979; Rowe, 2004; San Juan, 1995; Steffens, 1995).This might allow to tench to be ready to copping this situation, through the reduction of amonniun producction, through glutamine production is one possible strategy.

From a general point of view, the results show a strong interaction between tench and H-30 diet. In trout AST Km tend to increase with the *H. illucens* meal while in gilthead seabream increase when *T. molitor* were include. These difference seem to be consequence of the different metabolic adaptations of each specie, as well with the diet amino acids contribution and bioavailability (related with amino acids profile of the and digestibility of the source).

In summary, these data show differences in amino acid catabolism among the three species studied consistent with their nutritional requirements and feeding habits. Insect meal inclusion tended to increase amino acid catabolism in trout but not in gilthead seabream or tench. This is probably related to an imbalance in EAA/NEAA in the trout while gilthead seabream adapts the amino acids catabolism deviate more amino acids for glucose production and decreasing the amino acids used to energy production. In tench none effect was found except H-30 diet. These data suggest that the amino acids catabolism responds in each fish species depending on their nutritionals needs and the amino acids availability of each insect species.. More detailed studies are required to determine the nutritional utilization of amino acids from insect protein to improve insect meal performance in aquafeed.

Declaration of Competing Interest

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

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CAPÍTULO II: *Effect of feeding with insect meal diet on the fatty acid compositions of sea bream (*Sparus aurata*), tench (*Tinca tinca*) and rainbow trout (*Oncorhynchus mykiss*) fillets*

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Effect of feeding with insect meal diet on the fatty acid compositions of sea bream (*Sparus aurata*), tench (*Tinca tinca*) and rainbow trout (*Oncorhynchus mykiss*) fillets

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ABSTRACT

Currently, insects are considered a new source of food that could be an alternative to fishmeal (FM) in aquaculture. However, more studies are needed to assess the effects of the inclusion of insects in fish diets on both fish productivity and fish quality. To this end, three trials were conducted to evaluate as feed ingredients the meals of two insects, black soldier fly (*Hermetia illucens*, HI) and mealworm (*Tenebrio molitor*, TM), on the fatty acid (FA) profiles of fillets of three fish species: sea bream (*Sparus aurata*), tench (*Tinca tinca*) and rainbow trout (*Oncorhynchus mykiss*). In each trial, the animals were randomly divided into five experimental groups (3 tanks/treatment). The fish were fed until triple their initial weight with isoprotein and isolipid diets at different levels of FM replacement using insect meal: 0% insect meal or control diet (C), 15% inclusion of HI meal (H15), 30% inclusion of HI meal (H30), 15% inclusion of TM meal (T15), and 30% inclusion of TM meal (T30).

Overall, the use of insect meal induced a decrease in valuable long chain polyunsaturated fatty acids (LCPUFAs) in fish fillets, and the higher the insect inclusion (H30 and T30 diets), the higher the decreases in EPA and DHA contents. Moreover, insect-containing diets worsened the lipid health indices (*n*-3/*n*-6 ratio, atherogenicity AI, and thrombogenicity TI indices) of fillets. However, differences between the three fish species were observed: tench was the more resilient species to insect inclusion, while in rainbow trout, there were very marked decreases in LCPUFA content, especially in DHA, and in the *n*-3/*n*-6 ratio. Nevertheless, insect-fed fish could be considered "healthy" based on the *n*-3 LCPUFA content. Furthermore, several strategies could be implemented to avoid the decline of *n*-3 LCPUFAs in fish, such as using partially defatted insect meal or the use of insects previously fed *n*-3 PUFA-rich by-products.

1. Introduction

In the period 2000–2016, world food fish aquaculture production expanded at a rate of 5.8% per year (FAO, 2018), and since the world human population is continuously growing, it is expected that this trend will continue. Considering both the biological quality of the protein and its digestibility, fish meal (FM) is the ideal ingredient to feed fish. However, to base the growth of aquaculture on FM is unsustainable given that fish stocks are declining due to overfishing, pollution, climate change, and other factors, which has forced the search for alternative protein sources. To date, proteins of vegetable origin are the most commonly used alternatives to FM. Although these materials have been widely used, they show certain drawbacks, e.g., anti-nutritional components and/or some aminoacyl limitations (e.g., methionine, lysine and tryptophan) (Tacon, 1993).

In the context of resource scarcity and population growth, insect meals could represent a suitable alternative to FM in aquaculture feeds (Henry et al., 2015). Insects are a common food resource for omnivorous and carnivorous fish, particularly during the larval and fry stages (Nogales-Mérida et al., 2019). There are already numerous feeding trials

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where FM has been partially replaced by insect meal with promising results (e.g., Belghit et al., 2018; Iaconisi et al., 2017, 2018; Rahimnejad et al., 2019; Sankian et al., 2018). The mass production of insects has not yet been optimized, resulting in uncompetitive prices for insect meal, but with the support of the FAO and new legislation (including those of the EU) on sustainable foods, insect production systems can be expected to develop quickly, and prices could fall drop significantly in the coming years.

In aquaculture, it is a priority to produce feed to grow fish properly; another important task is the quality of the production. A drawback of using insects in aquaculture is that such animals often accumulate large amounts of fat, especially at immature stages (Henry et al., 2015). The fat content of insects varies greatly, between 10 and 60%, and such content is usually lower in adults than in larvae or pupae (Xiaoming et al., 2010). Most insect lipids, up to 80%, are stored to be used in times of high energy demand, such as egg development, hibernation or locomotive activity (Ruiz-Gutierrez and Pérez-Camino, 2000). Some species show high fat content which is inconvenient for the feeding of fish, due to the lipid quality requirements of fish; however, according to Defoliart (1991), the degree of unsaturation of the FA content in insects is similar to those of poultry and fish, which allows the percentage of substitution to be increased. However, the major disadvantage is that terrestrial insects lack *n*-3 LCPUFAs, such as eicosapentaenoic acid (20:5*n*-3, EPA) and docosahexaenoic acid (22:6*n*-3, DHA) (Sánchez-Muros et al., 2014). Therefore, knowing the effects of the dietary inclusion of insects in the FA profiles of fish fillets is an appropriate task. This is because fish, in addition to containing high-quality protein, constitute a raw source of healthy *n*-3 LCPUFAs. Thus, the quality of the lipid profiles of fish from aquaculture should be carefully checked.

n-3 PUFAs play an important role in human health, such as the prevention of certain types of cancer, joint pain, brain function during ageing and psychiatric diseases (Dillon et al., 2013). According to several studies, the intake levels of EPA and DHA are especially relevant (Din et al., 2004; Simopoulos, 2009; Uauy and Valenzuela, 2000). A diet rich in EPA and DHA prevents coronary heart disease, cancer, and autoimmune and inflammatory diseases (Gebauer et al., 2006; Robinson and Stone, 2006; Simopoulos, 2009; Trautwein, 2001). However, not only the amount of *n*-3 but also the ratio between *n*-6 and *n*-3 is important. According to Simopoulos (2002), Western diets tend to have high levels of *n*-6 and low levels of *n*-3 PUFAs. *n*-3 PUFAs are synthesized de novo by microalgae, and through the trophic chain, such PUFAs accumulate selectively in fish tissues (Gladyshev et al., 2013). Given that *n*-3 LCPUFAs cannot be synthesized by fish, their diet should include such compounds (Barcelo-Coblijn and Murphy, 2009).

To date, the feeding potential of some insect species has been evaluated, and in this experiment, we have focused on black soldier fly (*Hermetia illucens*, HI) and mealworm (*Tenebrio molitor*, TM) because, they have short cycles, are easy to feed and can be bred in massive quantities. Both HI (Kroeckel et al., 2012; Lock et al., 2016; Renna et al., 2017; Sealey et al., 2011; St-Hilaire et al., 2007; among others) and TM (Gasco et al., 2016; Iaconisi et al., 2017; Piccolo et al., 2017; Sánchez-Muros et al., 2017; Tubin et al., 2019, among others) are among the most evaluated insect meals in aquaculture.

Previous studies demonstrated that the inclusion of low amounts of HI or TM does not affect the production rates compared to FM-based diets; however, when assessing the quality of the fillet, FA profiles are a priority. As fish are monogastric animals, their FA profiles are highly influenced by the FA composition of the diets. In addition, differences in the FA profiles among different fish species fed the same diet could be expected, which is due to different feeding habits, the way of accumulating fat reserves and differences in the de novo synthesis of FA. In this context, this study aimed to assess the effects of partial FM replacements with two insect meals (HI and TM) at two different levels on the FA profiles of three aquaculture fish species (sea bream, tench and rainbow trout).

2. Materials and methods

2.1. Diets

HI and TM larvae meal used in this study were purchased from Entomotech S.L. (Almería, Spain) and MealFood Europe S.L. (Salamanca, Spain), respectively. Five experimental diets were formulated to be isoproteic (46%) and isolipidic (17%): a diet control with 0% insect meal, two levels of HI larvae meal in substitution of 15% (H15) and 30% (H30) FM, and two levels of TM larvae meal in substitution of 15% (T15) and 30% (T30) FM. The ingredients of the experimental diets are reported in Table 1. The diets were manufactured by Life BIO-ENCAPSULATION S.L. (Almería, Spain).

2.2. Growth trial

The fish used for this experiment were juveniles of three different species: sea bream (*Sparus aurata*), tench (*Tinca tinca*) and rainbow trout (*Oncorhynchus mykiss*). Sea bream (450 animals) with an initial weight of 22 g each were supplied by Predomar S.L. (Carboneras, Almería) and were raised in facilities at Almería University. A total of 600 rainbow trout with an initial weight of 55 ± 0.7 g each, supplied by a commercial farm (Piscifactoría Fuente del Campillo, Guadalajara, Spain), were raised at the experimental facilities of the Aquaculture Research Centre of "Instituto Tecnológico Agrario de Castilla y León" (ITACyL). A total of 3000 tench with an initial weight of 15 g each were raised at the Aquaculture Centre of Vegas del Guadiana (from Junta de Extremadura). All fishes were fed commercial feed for acclimation for 15 days before the growth trial, and then, the fish were individually weighed and randomly divided into tanks (three replicate tanks per diet). During the experimental period, the fish were kept in constant temperature conditions (20 ± 1 °C for sea bream, 15 ± 1 °C for rainbow trout and 22 ± 1 °C for tench) and natural photoperiod for sea bream, 12 h light:12 h dark for rainbow trout, and 14 h light:10 h dark for tench. The animals were

Table 1
Ingredients and proximate compositions of HI and TM larvae meals and experimental diets.

	Experimental diets						
	Control	H15	H30	T15	T30	HI	TM
Ingredients (g kg^{-1} dry weight)							
Fishmeal	368	315	259	313	259		
Hermetia illucens	0	56	109	0	0		
Tenebrio molitor	0	0	0	51	107		
Wheat gluten	105	127	148	121	126		
Soy protein concentrate	151	159	170	151	165		
Wheat meal	162	140	119	156	146		
Soy lecithin	13	10	10	10	8		
Fish oil	117	108	98	113	107		
Vitamins and minerals	20	20	20	20	20		
Goma guar	20	20	20	20	20		
Hemoglobin	39	39	39	39	39		
Methionine	0,8	1,5	2,2	1,5	2,2		
Lysine	5,6	5,8	6	5,9	6,1		
Proximate composition (% dry weight)							
Crude protein	45.8	46.6	45.4	46.5	46.3	40.1	55.3
Ether extract	16.5	17.4	17.3	17.4	16.8	33.9	28.3
Crude fiber	1.5	1.9	1.5	1.3	1.9		
Chitin						16.6	5.9
Ash	8.1	7.9	7.8	7.5	8.2	10.6	3.7
Gross energy (Kj g − 1)	16.8	16.9	17.0	16.8	16.8		

H15 and H30: 15% and 30% fishmeal replacement with HI, respectively; T15 and T30: 15 and 30% fishmeal replacement with TM, respectively; HI *Hermetia illucens*,; TM *Tenebrio molitor*. Gross energy was calculated based on 1 g crude protein being 23.6 Kj, 1 g crude fat being 39.5 Kj and 1 g carbohydrate being 17.2 Kj (National Research Council, 2011).

fed ad libitum twice a day. The daily intake was recorded, and the feeding trial concluded when fish tripled their initial weight, approximately 45 days in sea bream, 46 days in rainbow trout and 100 days in tench.

2.3. Samples

At the end of the experiment, several fish were randomly sampled from each tank and sacrificed by administering an overdose of anaesthesia (MS-222, 300 mg·mL^{-1}), until no opercular movements were detected for 2 min, and fillet samples were collected for their corresponding analysis. The samples were frozen in liquid N_2 and stored at −80 °C for subsequent analysis.

2.4. Chemical analyses

The nutritional profiles of insect meals and experimental diets were characterized according to the standard procedures of the Association of Official Analytical Chemists (AOAC, 2005), with specific methods as follows: the crude protein (CP) content was determined by Kjeldahl * 625 (AOAC, 2005; #954.01) (Nx6.25), ether extract (EE) was determined by ethyl ether extraction (Soxhlet technique) (AOAC, 2005; #920.39), moisture was determined gravimetrically by drying at 105 ± 0.5 °C (AOAC, 2005; #934.01), an enzymatic–gravimetric method was used to determine the crude fiber (AOAC, 2005; #985.29), and ash were determined gravimetrically after combustion at 500 °C in a muffle furnace (AOAC, 2005; #942.05) to a constant weight. The chitin was determined according to the method described by Gamage and Shahidi (2007). All analyses were performed in triplicate. The proximate compositions of both HI and TM larvae meal and of the experimental diets are shown in Table 1.

2.5. FA analyses

FA determination in samples was carried out after direct derivatization to FA methyl esters (FAMEs) (Lepage and Roy, 1984; Rodríguez-Ruiz et al., 1998). Fifty milligrams of freeze-dried sample was placed in test tubes and mixed with 1 mL of methylating mixture (20:1 v/v, methanol:acetyl chloryde) and 1 mL of n-hexane. Test tubes were capped and heated for 30 min at 100 °C. The tubes were cooled to room temperature, and 1 mL of distilled water was added. The tubes were centrifuged (2000 ×*g*, 5 min), and the organic phase was collected for GC-FID analysis. FAMEs were analysed in a Focus GC (Thermo Electron. Cambridge. UK) equipped with a flame ionization detector (FID) and an Omegawax 250 capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Supelco, Bellefonte, USA), as previously described (Guil-Guerrero et al., 2014). The peak area of the internal standard (nonadecanoic acid, 0.5 mg/mL) was used as a reference to calculate the mass of each FA in the resulting chromatograms. The relative retention factors for each FA as reported by Cladis et al. (2014) were considered for quantification. The FA compositions of both HI and TM larvae meal are shown in Table 2.

2.6. Indices of lipid nutritional quality

In addition to the FA profiles of the fillets, the nutritional quality of the lipid fraction was assessed by considering the following indices: the PUFA *n*-3/PUFA *n*-6 ratio and the atherogenicity (AI) and thrombogenicity (TI) indices, according to the following calculations:

a) Atherogenicity index (Ulbricht and Southgate, 1991).

$$AI = [12:0 + (4 \times 14:0) + 16:0]/(\Sigma n-3\ \text{PUFA} + \Sigma n-6\ \text{PUFA} + \Sigma\text{MUFA})$$

Table 2
Fatty acid compositions (g/100 g of total fatty acids) of both HI and TM larvae meals.

	HI	TM
10:0	1.19	n.d.
12:0	42.91	n.d.
14:0	8.33	2.39
16:0	14.74	17.81
16:1*n*7	2.44	1.41
18:0	2.69	3.48
18:1*n*9	15.35	34.57
18:1*n*7	n.d.	n.d.
18:2*n*6	8.87	37.47
18:3*n*3	0.57	1.70
18:4*n*3	0.98	n.d.
20:1*n*9	n.d.	n.d.
20:5*n*3	n.d.	n.d.
22:1*n11*	n.d.	n.d.
22:5*n*3	n.d.	n.d.
22:6*n*3	n.d.	n.d.
SFA	69.86	23.68
MUFA	17.79	35.99
PUFA	10.42	39.17
n-3	1.54	1.69
n-6	8.87	37.47
n-3/n-6	0.17	0.05

HI *Hermetia illucens*; TM *Tenebrio molitor*; n.d.: not determined; SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids.

b) Thrombogenicity index (Ulbricht and Southgate, 1991).

$$TI = (14:0 + 16:0 + 18:0)/[(0.5 \times \Sigma\text{MUFA}) + (0.5 \times \Sigma n-6\ \text{PUFA}) + (3 \times \Sigma n-3\ \text{PUFA}) + (\Sigma n-3\ \text{PUFA}/\Sigma n-6\ \text{PUFA})]$$

2.7. Statistical analysis

Because the data were not adjusted to the general linear model (ANOVA) requirements, such as normal distribution and homogeneity of variance, generalized linear models (GZLMs) were used to examine the functional relationships between FA composition obtained from fish fillets (response variable) and the different experimental diets (explanatory variables). The models were fitted by a maximum quasi-likelihood estimation with the GenLin procedure with gamma errors and the logarithm link function using the IBM SPSS version 25.0 statistical software package (IBM, 2015). In each trial, the significance of the model was assessed by an omnibus test (to test whether the explained variance in a data set is significantly greater than the unexplained variance). For each regression effect specified in the model, a Wald statistic (Wald chi2) was conducted, which is a test based on linearly independent pairwise comparisons among the estimated marginal means. The mean values were then compared pairwise, with significance indicated at $P = 0.05$ using the same statistical software (IBM, 2015).

2.8. Ethical standards

The experiment was conducted according to the directive 2010/63/EU of the European Parliament for the protection of animals used in experiments and for other scientific purposes and was approved by the Bioethics Committee of the University of Almeria according to Royal Decree 53/2013 of 1 February 2013.

3. Results

3.1. Effects on feed

As summarized in Table 2, the two insect larvae used in this test show very different FA profiles (FA% of total FA). In TM, both oleic acid (OA, 18:1*n*-9) (34.6%) and linoleic acid (LA 18:2*n*-6) (37.5%) stood out,

while in HI, they had lower percentages: 15.3% and 8.9%, respectively. In contrast, lauric acid (LaA, 12:0) reached higher amounts in HI (42.9%), while TM lacked it. Notably, EPA and DHA were not detected in either insect.

Such differences in the FA profiles of the insect meals induced significant differences in the results of feeding trials (Table 3). Consequently, LaA occurred in feeds H15 (5.6%) and H30 (10.5%), while it was not detected in the remaining diets. Additionally, feeds including TM meal (T15 and T30) showed higher percentages of OA and LA. As a general trend, as the dietary inclusion of insects increased, EPA and DHA decreased.

In HI meal (Table 2), saturated fatty acids (SFAs) reached high figs. (69%) when compared to TM meal (23.7%), mainly due to the presence of LaA (42.9%), although it also contained some amounts of myristic acid (MA, 14:0) (8.33%). Therefore, SFAs were proportionally higher in feeds H15 and H30 (Table 3). Moreover, TM meal contained more PUFAs (39%, mainly LA (37.5%) than HI meal (10.4%); thus, TM induced high PUFA percentages in the feed. Both HI and TM contained low percentages of *n*-3 PUFAs; thus, feeds based on such insects will always contain lower proportions of such PUFAs than the control feed, especially the H30 and T30 feeds, which had the highest percentages of insect meal. Finally, as expected, the *n*-3/*n*-6 ratio decreased in insect feeds (Table 3) and to a greater extent in those containing TM.

Table 3
Fatty acid compositions (g/100 g of total fatty acids) of experimental diets.

	Experimental diets				
	Control	H15	H30	T15	T30
12:0	n.d.	5.22 ± 0.06^{b}	9.60 ± 0.11^{a}	n.d.	n.d.
14:0	4.94 ± 0.03^{c}	5.20 ± 0.03^{b}	5.44 ± 0.03^{a}	4.63 ± 0.02^{d}	4.46 ± 0.02^{e}
16:0	19.30 ± 0.18^{a}	17.92 ± 0.17^{c}	17.80 ± 0.17^{c}	18.71 ± 0.17^{b}	19.02 ± 018^{ab}
16:1*n*7	5.00 ± 0.03^{a}	4.69 ± 0.03^{b}	4.42 ± 0.03^{c}	4.74 ± 0.03^{b}	4.41 ± 0.03^{c}
17:0	0.78 ± 0.00^{a}	0.69 ± 0.00^{c}	0.61 ± 0.00^{e}	0.71 ± 0.00^{b}	0.65 ± 0.00^{d}
18:0	4.08 ± 0.01^{a}	3.71 ± 0.01^{c}	3.56 ± 0.01^{d}	3.89 ± 0.01^{b}	3.91 ± 0.01^{b}
18:1*n*9	14.38 ± 0.04^{d}	14.56 ± 0.04^{c}	14.59 ± 0.04^{c}	16.33 ± 0.05^{b}	18.15 ± 0.06^{a}
18:1*n*7	2.77 ± 0.01^{a}	2.46 ± 0.01^{c}	2.26 ± 0.01^{e}	2.56 ± 0.01^{b}	2.30 ± 0.01^{d}
18:2*n*6	9.39 ± 0.04^{e}	9.64 ± 0.04^{d}	10.68 ± 0.05^{c}	12.06 ± 0.05^{b}	14.97 ± 0.06^{a}
18:3*n*3	1.63 ± 0.00^{c}	1.52 ± 0.0^{d}	1.49 ± 0.00^{e}	1.64 ± 0.00^{b}	1.67 ± 0.00^{a}
18:4*n*3	1.51 ± 0.00^{a}	1.34 ± 0.00^{c}	1.16 ± 0.00^{e}	1.39 ± 0.00^{b}	1.25 ± 0.00^{d}
20:1*n*9	3.03 ± 0.04^{a}	2.82 ± 0.03^{b}	2.24 ± 0.03^{e}	2.66 ± 0.03^{c}	2.37 ± 0.03^{d}
20:4*n*6	1.02 ± 0.00^{a}	0.91 ± 0.00^{c}	0.81 ± 0.00^{e}	0.94 ± 0.00^{b}	0.85 ± 0.00^{d}
20:4*n*3	0.54 ± 0.00^{a}	0.49 ± 0.00^{c}	0.43 ± 0.00^{e}	0.50 ± 0.00^{b}	0.46 ± 0.00^{d}
20:5*n*3	8.89 ± 0.03^{a}	7.95 ± 0.03^{c}	6.97 ± 0.02^{e}	8.24 ± 0.03^{b}	7.42 ± 0.02^{d}
22:1*n11*	3.70 ± 0.02^{a}	3.34 ± 0.02^{c}	2.93 ± 0.02^{e}	3.47 ± 0.02^{b}	3.01 ± 0.02^{d}
22:5*n*3	1.34 ± 0.02^{a}	1.16 ± 0.02^{b}	0.99 ± 0.02^{d}	1.20 ± 0.02^{b}	1.08 ± 0.02^{c}
22:6*n*3	11.84 ± 0.18^{a}	10.60 ± 0.16^{b}	9.01 ± 0.14^{d}	10.91 ± 0.17^{b}	9.68 ± 0.15^{c}
24:1*n*9	0.46 ± 0.01^{a}	0.39 ± 0.01^{b}	0.33 ± 0.00^{d}	0.40 ± 0.01^{b}	0.36 ± 0.00^{c}
SFA	29.12 ± 0.21^{c}	32.73 ± 0.23^{b}	37.01 ± 0.26^{a}	27.95 ± 0.20^{d}	28.04 ± 0.20^{d}
MUFA	29.25 ± 0.06^{c}	28.25 ± 0.06^{d}	26.77 ± 0.05^{e}	30.15 ± 0.06^{b}	30.69 ± 0.06^{a}
PUFA	36.17 ± 0.15^{c}	32.62 ± 0.14^{d}	31.54 ± 0.13^{e}	36.89 ± 0.16^{b}	37.36 ± 0.16^{a}
n-3	25.76 ± 0.20^{a}	23.01 ± 0.18^{c}	20.05 ± 0.16^{e}	23.88 ± 0.19^{b}	21.55 ± 0.17^{d}
n-6	10.40 ± 0.42^{e}	10.56 ± 0.04^{d}	11.49 ± 0.05^{c}	13.01 ± 0.05^{b}	15.81 ± 0.06^{a}
n-3/n-6	2.48 ± 0.03^{a}	2.18 ± 0.02^{b}	1.75 ± 0.02^{d}	1.84 ± 0.02^{c}	1.36 ± 0.01^{e}

HI *Hermetia illucens*; TM *Tenebrio molitor*; H15 and H30: 15% and 30% fishmeal replacement with HI, respectively; T15 and T30: 15 and 30% fishmeal replacement with TM, respectively. n.d.: not determined; SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids. Different lowercase letters indicate significant differences ($P \leq 0.05$) between experimental diets.

3.2. Intraspecies effects

The change produced in the FA profiles of fish fillets by the inclusion of insect meal in feeds was lower in tench than in the other assessed fishes (Table 4). In this way, the fillets of sea bream and rainbow trout experienced significant changes in their FA profiles when reared using feed containing insect meal.

As LaA was higher in HI-based feeds, this FA only appeared in the fillets of the three fishes fed with H15 and H30 diets. MA was significantly higher in fish fed H15 and H30 in sea bream and rainbow trout than in fish fed the control diet, but no significant differences were observed in tench. Palmitic acid (PA, 16:0) decreased in sea bream when TM meal was included. Rainbow trout showed a lower amount of stearic acid (SA, 18:0) in fillets of samples fed TM (T15 and T30). As a result, individuals fed HI, regardless of fish species, had significantly higher proportions of SFAs.

Other interesting changes were observed in the relative OA percentages in rainbow trout fillets fed insect meal diets, especially using T15 and T30 feeds. However, in sea bream, only the T30 diet significantly increased the content of OA in fillets. As a result, in rainbow trout, fish fed the TM feed had the highest monounsaturated fatty acid (MUFA) content. Similarly, LA increased in the muscles of fish fed insect feeds, and a higher content was obtained using the T30 diet, although this tendency was observed in the three fish species.

The main drawback derived from the inclusion of insects in feeds is that there were decreases in valuable *n*-3 PUFAs in fillets, such as EPA and DHA. In sea bream and rainbow trout, there was an inverse relationship between the proportion of EPA and the degree of inclusion of insects in feeds; thus, the lowest content in fillets coincides with the maximum content of insect meal (H30 and T30), although this tendency was not observed in tench. Regarding DHA, only in the fillets of rainbow trout fed the insect feeds was a significant decrease in this PUFA observed, and this decrease was especially marked in the two TM feeds (T15 and T30). Overall, *n*-3 VLCPUFAs decreased significantly in both sea bream and rainbow trout, and T30 feed induced the lowest contents.

With regard to the indices of the nutritional quality of lipids, in general, by using insect-based feeds, both HI and TM showed significant decreases in the *n*-3/*n*-6 ratio in fish fillets, and in the three fish species, the lowest *n*-3/*n*-6 ratio was observed when feeding the T30 feed. While AI increased using H15 and H30 diets, mainly due to the high LaA content of HI, TI increased significantly in sea bream and rainbow trout at the highest level of insect inclusion (both H30 and T30).

3.3. Interspecies effect

Fig. 1 shows the changes in the *n*-3 PUFA profiles of the three fish species due to the dietary inclusion of HI and TM in feeds. Overall, the relative amount of α-linolenic acid (ALA, 18:3*n*-3) was low, and it was much higher in control-fed rainbow trout (1.9%) than in tench (1.3%) and sea bream (1.1%) (Fig. 1a). Considering insect meal-reared fish, in rainbow trout, the ALA percentage increased using both the T15 and T30 diets, while in sea bream, the effect was the opposite, decreasing with the T30 diet. In tench, all insect-based feeds decreased the content of ALA in the fillet, and the diets with HI caused the greatest decrease.

Fig. 1b shows changes in the EPA% of total FA for the three fish

Table 4
Effects of the dietary inclusion of HI and TM larvae meal on fatty acid profiles (g/100 g of total fatty acids) of sea bream, tench and rainbow trout fillet muscle.

	Control	H15	Sea bream	T15	T30	Control	H15	Tench	T15	T30	Control	H15	Rainbow trout		
			H30					H30					H30	T15	T30
12:0	n.d.	2 ± 0.1	3.5 ± 0.1	n.d.	n.d.	n.d.	1 ± 0.1	2.2 ± 0.2	n.d.	n.d.	n.d.	1.7 ± 0.1	3.3 ± 0.1	n.d.	n.d.
14:0	3.3 ± 0.1^{b}	4.1 ± 0.1^{a}	4.5 ± 0.2^{a}	3.3 ± 0.1^{b}	3.1 ± 0.1^{b}	2.8 ± 0.3	2.4 ± 0.2	2.7 ± 0.3	2.4 ± 0.2	2.5 ± 0.2	3 ± 0.1 ^{c}	3.5 ± 0.1 ^{ab}	3.8 ± 0.2 ^{a}	3.2 ± 0.1 ^{bc}	3.2 ± 0.1 ^{bc}
16:0	18.5 ± 0.3^{a}	17.9 ± 0.3^{ab}	18 ± 0.3^{ab}	17.3 ± 0.3^{b}	17.4 ± 0.3^{b}	19.5 ± 0.5	20.6 ± 0.5	20.3 ± 0.5	20.8 ± 0.5	19.1 ± 0.5	17.1 ± 0.3	17 ± 0.3	16.9 ± 0.3	16.3 ± 0.2	15.7 ± 0.3
16:1n7	5.0 ± 0.1^{a}	5.1 ± 0.1^{a}	5 ± 0.1^{a}	4.7 ± 0.1^{ab}	4.5 ± 0.1^{b}	6.7 ± 0.6	5.1 ± 0.4	5.1 ± 0.4	5.8 ± 0.5	6.2 ± 0.5	3.7 ± 0.2	3.8 ± 0.2	3.9 ± 0.2	4.1 ± 0.2	4 ± 0.2
17:0	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	0.6 ± 0.0^{b}	0.6 ± 0.0^{a}	0.5 ± 0.0^{b}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18:0	4.5 ± 0.2	4.2 ± 0.2	4.2 ± 0.2	4.3 ± 0.2	4.5 ± 0.2	3.6 ± 0.4	4.7 ± 0.5	4.5 ± 0.5	4.3 ± 0.4	3.6 ± 0.4	4.2 ± 0.1 ^{a}	3.9 ± 0.1 ^{ab}	3.9 ± 0.1 ^{ab}	3.7 ± 0.1 ^{bc}	3.6 ± 0.1 ^{c}
18:1n9	20.8 ± 0.4^{b}	20.4 ± 0.4^{b}	20.8 ± 0.4^{b}	20.9 ± 0.4^{b}	22.9 ± 0.4^{a}	20.2 ± 0.9	18.2 ± 0.8	18.2 ± 0.8	19.9 ± 0.9	21.7 ± 1	19.8 ± 0.9 ^{d}	22.0 ± 1.0 ^{cd}	22.9 ± 1.2 ^{bc}	26.2 ± 1.1 ^{b}	27.4 ± 1.3 ^{a}
18:1n7	3.3 ± 0.0^{a}	3 ± 0.0^{b}	2.8 ± 0.0^{c}	3.1 ± 0.0^{b}	2.8 ± 0.0^{c}	3.1 ± 0.1	2.7 ± 0	2.6 ± 0	2.8 ± 0	2.8 ± 0	2.9 ± 0.1	2.8 ± 0.1	2.7 ± 0.1	2.8 ± 0.1	2.6 ± 0.1
18:2n6	8.3 ± 0.2^{e}	9.3 ± 0.2^{d}	10.2 ± 0.2^{c}	11.2 ± 0.2^{b}	12.9 ± 0.3^{a}	8.1 ± 0.6 ^{b}	7.2 ± 0.5 ^{b}	7.8 ± 0.6 ^{b}	8.6 ± 0.6 ^{b}	10.9 ± 0.8 ^{a}	9.8 ± 0.4 ^{d}	10.6 ± 0.4 ^{cd}	11.8 ± 0.6 ^{bc}	13.3 ± 0.5 ^{b}	15 ± 0.7 ^{a}
18:3n6	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18:3n3	1.1 ± 0.0^{a}	1.1 ± 0.0^{a}	1.0 ± 0.0^{ab}	1.0 ± 0.0^{a}	0.9 ± 0.0^{b}	1.3 ± 0.1^{a}	0.7 ± 0.1^{cd}	0.6 ± 0.1^{d}	0.9 ± 0.1^{bc}	1 ± 0.1^{b}	1.9 ± 0.1^{c}	1.9 ± 0.1^{c}	1.9 ± 0.1^{bc}	2.1 ± 0.1^{ab}	2.2 ± 0.1^{a}
18:4n3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.9 ± 0	0.9 ± 0	0.9 ± 0	0.9 ± 0	0.9 ± 0
20:1n9	2.7 ± 0.2^{a}	2.3 ± 0.1^{abc}	2.2 ± 0.1^{b}	2.4 ± 0.1^{ab}	2 ± 0.1^{c}	2.6 ± 0.2	1.8 ± 0.1	1.6 ± 0.1	2.2 ± 0.1	2.3 ± 0.2	2.2 ± 0.2	2.2 ± 0.2	2.3 ± 0.2	3 ± 0.2	2.9 ± 0.2
20:4n6	1.1 ± 0.1	1 ± 0.1	1 ± 0.1	1.1 ± 0.1	1 ± 0.1	1.8 ± 0.2	2.5 ± 0.3	2.3 ± 0.3	2 ± 0.3	1.9 ± 0.2	1.1 ± 0.1	1 ± 0	0.9 ± 0.1	0.8 ± 0	0.7 ± 0
20:4n3	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	n.d.	n.d.	n.d.	n.d.	n.d.	0.7 ± 0	0.7 ± 0	0.6 ± 0	0.7 ± 0	0.7 ± 0
20:5n3	6.9 ± 0.2^{a}	6.4 ± 0.2^{ab}	6.0 ± 0.2^{bc}	6.4 ± 0.2^{ab}	5.7 ± 0.2^{c}	7 ± 0.3	6.9 ± 0.3	6.5 ± 0.2	6.8 ± 0.2	6.1 ± 0.2	6.0 ± 0.2 ^{a}	5.3 ± 0.2 ^{b}	4.6 ± 0.2 ^{c}	4.6 ± 0.2 ^{c}	4.1 ± 0.2 ^{d}
22:1n11	2.2 ± 0.1	2 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	1.8 ± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
22:5n3	2.4 ± 0.1^{a}	2.2 ± 0.1^{b}	2.0 ± 0.1^{c}	2.2 ± 0.1^{b}	1.9 ± 0.1^{c}	2 ± 0.1	2 ± 0.1	2.1 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	2 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
22:6n3	14 ± 0.7	13 ± 0.6	12.2 ± 0.6	13.6 ± 0.7	11.8 ± 0.6	19.2 ± 1.7	23 ± 2	21.9 ± 1.9	20.9 ± 1.8	18.3 ± 1.6	22.8 ± 1.6^{a}	18.9 ± 1.4^{ab}	16.2 ± 1.4 ^{bc}	13.3 ± 1.0 ^{cd}	11.5 ± 0.9 ^{d}
24:1n9	0.5 ± 0	0.4 ± 0	0.4 ± 0	0.5 ± 0	0.4 ± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

HI: *Hermetia illucens*; TM: *Tenebrio molitor*; H15 and H30: 15% and 30% fishmeal replacement with HI, respectively; T15 and T30: 15 and 30% fishmeal replacement with TM, respectively; n.d.: not determined. Different lowercase letters indicate significant differences ($P \leq 0.05$) between experimental diets within each fish species (intra-species).

species. In the control group, the proportions of EPA in tench and sea bream were very similar (~7%) and slightly higher than that found in rainbow trout (6%). The substitution of FM by insect meal decreased the proportion of EPA, although this change was only significant in sea bream and rainbow trout. As expected, T30 feed induced the highest decreases: 5.7% in sea bream and 4.1% in rainbow trout.

Fig. 1c shows changes in DHA% of total FA in the fillet. It can be noted that the dietary inclusion of insect meal in feeds induced very different responses among fish species. Regarding the control diet, there were already large differences between the species: sea bream had the lowest percentage of DHA (14%), and rainbow trout had the highest percentage (23%). However, while there were no significant differences in DHA% in fillets when consuming insect-based meals in sea bream and tench, in rainbow trout, there was a marked decrease in DHA%, which was proportional to the degree of insect inclusion, with the T30 diet containing the lowest DHA%. This caused the rainbow trout to show no difference with tench fed the Control and H15 feeds, being in an intermediate position with the H30 feed, and containing a similar (and lower) proportion of sea bream with individuals fed the T15 and T30 diets.

The SFA content for the three fish species is given in Fig. 2a, which showed similar responses to changes of fishmeal by insect meal. For all diets, rainbow trout was the species that contained the lowest SFA amounts, while such contents were very similar in sea bream and tench. For all species, H30-fed fishes contained the highest percentages of SFAs, and T30-fed fishes contained the lowest percentages. Regarding MUFA (Fig. 2b) and PUFA (Fig. 2c) content, both tench and sea bream showed little variation when consuming insect meal-based diets. However, in rainbow trout fillets, MUFA proportions increased significantly using both T15 and T30 feeds. Control-fed rainbow trout contained the highest proportion of PUFAs (45%), and after consuming insect-based feeds, such content decreased markedly, especially using TM diets, equaling the proportion of sea bream (36%).

Fig. 3 shows the quality indices derived from the FA contents. As expected, insect meal-based feeds produced a significant decrease in the *n*-3/*n*-6 ratio in sea bream, and this was much higher in rainbow trout (Fig. 3a). While the *n*-3/*n*-6 ratio reached the highest figure in control-fed rainbow trout (3.3) and was lowest in control-fed sea bream (2.3), for TM-fed fishes, this ratio was always similar, showing that both species had the lowest values when feeding the T30 diet (1.3).

Fig. 3b shows AI changes in fillets derived from the inclusion of insect meal in feeds, which were very similar in the three fish species. AI increased significantly using H15 and H30 feeds, and H30 induced the highest variation. However, for TM-fed fishes, this ratio decreased significantly with respect to control fish, especially when using the T30 diet. With respect to TI (Fig. 3c), it increased in sea bream and rainbow

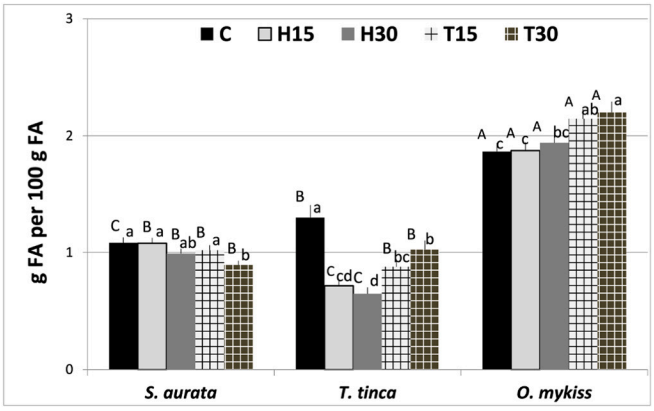

Fig.1a ALA (18:3n3)

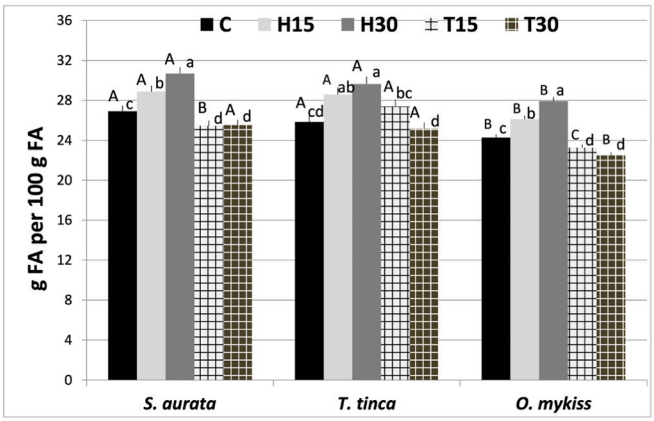

Fig.2a SFA

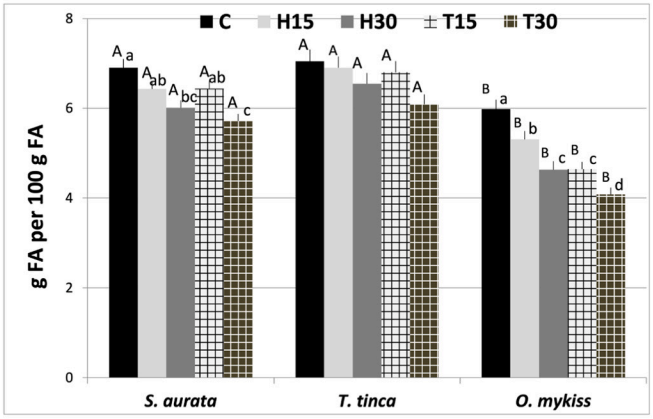

Fig.1b EPA (20:5n3)

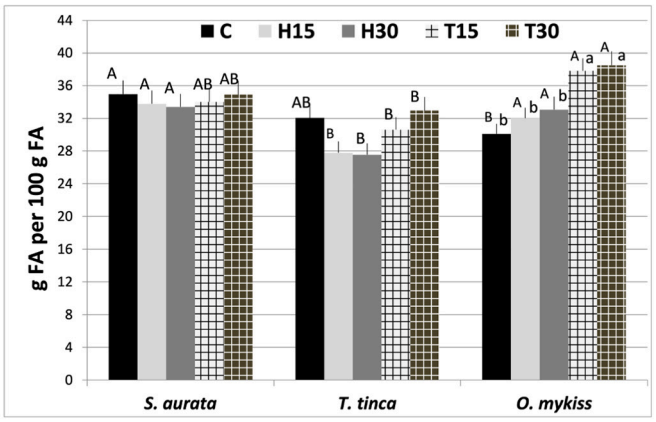

Fig.2b MUFA

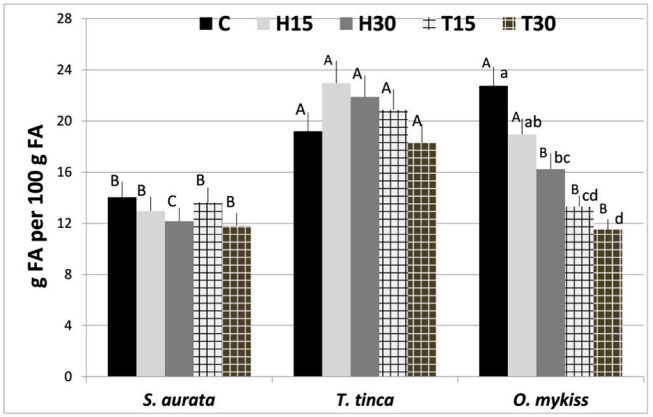

Fig.1c DHA (22:6n3)

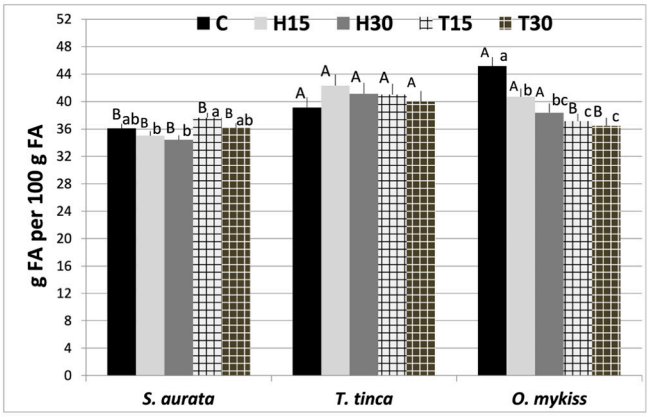

Fig.2c PUFA

Fig. 1. Differences in the effects of the dietary inclusion of HI and TM larvae meal on selected *n*-3 fatty acid profiles (g/100 g of total fatty acids) in fillet muscle between sea bream, tench and rainbow trout.

Fig. 2. Differences in the effects of the dietary inclusion of HI and TM larvae meal on the contents of the fatty acid groups (g/100 g of total fatty acids) in fillet muscle between sea bream, tench and rainbow trout.

trout, especially for fish reared at the highest ratio of insect inclusion in meals (H30 and T30 diets).

4. Discussion

In this paper, the FA profile of three species of fish fed insect meal was evaluated as fillet quality indicator. In a previous article, Fabrikov et al. (2020), with fish from the same experiment, found that there was almost no difference in growth and diet utilization. Thus, no differences were observed between the diets in the final weight of any species, and only in gilthead seabream fed with the H30 diet the percentage of weight gain was lower than in the other cases. In relation to diet utilization, rainbow trout fed with the T15 diet showed a lower FCR in comparison to those grown up with H15 and H30 diets. In tench, the inclusion of insect meal caused no effect on both weight gain or diet utilization.

4.1. Intraspecies effect

Insects have a great future as sustainable ingredients to replace fish meal in the aquaculture industry. However, more studies are needed to assess changes in the quality of fillets when including such ingredients in

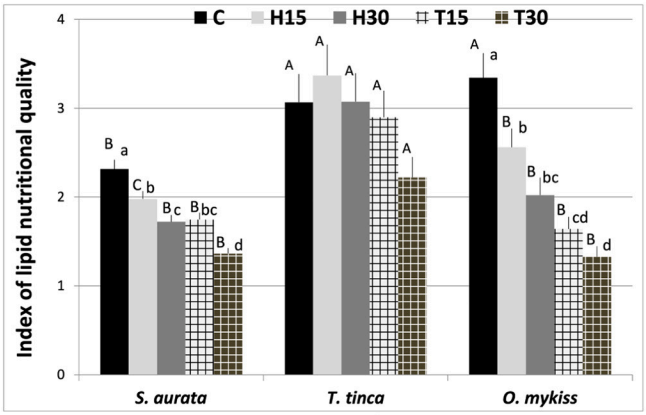

Fig.3a n-3/n-6

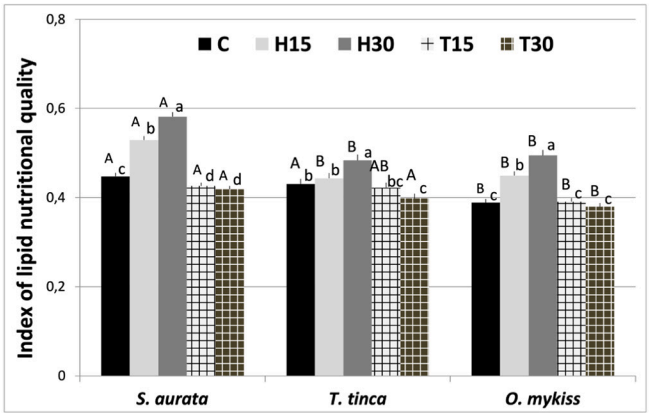

Fig.3b AI

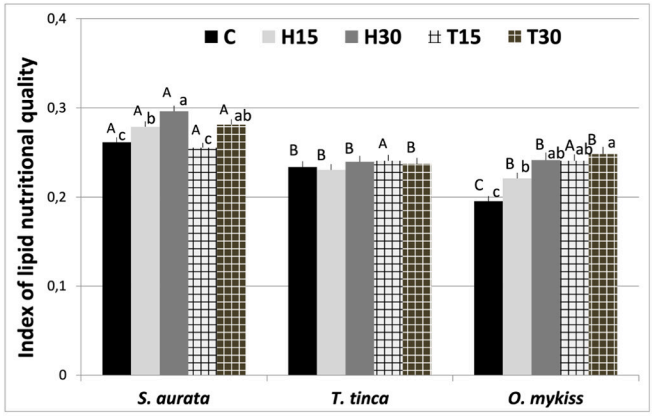

Fig.3c TI

Fig. 3. Differences in the effects of the dietary inclusion of HI and TM larvae meals on indices of lipid nutritional quality in fillet muscle between sea bream, tench and rainbow trout.
HI: *Hermetia illucens*; TM: *Tenebrio molitotor*; C: control diet; H15 and H30: 15% and 30% fishmeal replacement with HI, respectively; T15 and T30: 15 and 30% fishmeal replacement with TM, respectively; n.d.: Not determined; SFA saturated fatty acids; MUFA monounsaturated fatty acids; PUFA polyunsaturated fatty acids; AI: atherogenicity index; TI: thrombogenicity index. Different capital letters between bars indicate significant differences ($P \leq 0.05$) between the three species of fish (inter-species) for the same experimental diet. Different lowercase letters on the bar indicate significant differences ($P \leq 0.05$) between experimental diets within each fish species (intra-species).

fish feeds. There is a huge diversity of insect species in nature. They have evolved by adapting to different environments and needs and thus have developed different feeding habits, which have allowed them to subsist on different food resources. Therefore, the FA profiles of TM, specialized in the consumption of cereals, and of HI, a detritivore species, are very different.

The most abundant SFA in TM meal was PA (17.8%), while it was slightly less abundant in HI (14.7%) (Table 2). The most notable difference in the FA profiles between these two insect species was the high level of SFAs in HI meal (69.9%), while in TM meal it was only 23.7%, and such figures agree with those of Renna et al. (2017) and Nogales-Mérida et al. (2019). This high level is due to LaA content, close to 43% in HI, while TM lacks it.

This high content of LaA is an exclusive and common characteristic of HI (e.g., Nogales-Mérida et al., 2019; Oonincx et al., 2015, 2019). The high content of SFA of Hi is reflected in muscle of specimens feed with H15 and H30 diets. Similar tend was detected for LaA, which also accumulated only in the muscles of H15- and H30-fed specimens. Similar results were described by Renna et al. (2017), who found higher proportions of this FA in rainbow trout fed with 25%-HI diets (8%). As these authors indicated, the level of LaA in the fillets was much lower than that provided in the diet because it is used as an energy source. Then, the accumulation could be influenced by the energy content of the diet and the energy needs of animals.

OA was the most abundant MUFA in TM (34.6%), while in HI, it accounted for lower amounts (15.6%), probably due to their natural diet. Other authors found higher figures for OA in TM, ranging from 36 to 49% (Nogales-Mérida et al., 2019). Similar to what happened to LaA, OA accumulated in sea bream and rainbow trout fed with T15 an T30 diet (rich in OA) (Table 4), in agreement with previously described in rainbow trout and shrimp (*Litopenaeus vannamei*) (Belforti et al., 2015; Panini et al., 2017), while no significant differences were found in tench.

Although this FA is not essential for humans, consumption of OA has beneficial effects on health by lowering LDL cholesterol, among several other healthy effects (Aranceta and Gil, 2010).

According to Finke and Oonincx (2017), some insect fats contain high PUFA percentages of total FAs. Our results show a high LA and PUFAs proportion in TM, while HI contained a low percentage. However, neither HI nor TM contain *n*-3 LC PUFAs, such as EPA or DHA, and have low LA percentages.

When assessing the potential of a new ingredient in aquaculture, such as insects, in addition to optimal fish growth, it is necessary to guarantee adequate fish quality to consumers. As indicated above, *n*-3 PUFA consumption induces several health benefits (Orsavova et al., 2015). Given that HI and TM larvae do not contain either EPA or DHA, insect-based feeds contain lower amounts of VLCPUFAs, and consequently, the EPA levels obtained in sea bream and rainbow trout fillets were lower in insect-fed fish, especially at higher insect inclusion (H30 and T30). However, tench did not follow this expected trend due their capacity to biosyntethize DHA (Garrido et al., 2020). For DHA muscle levels, only HI-fed rainbow trout showed a decrease, and T30-fed samples contained half the DHA content compared to the control. However, regardless of the fish species and experimental feed examinated, the proportion of DHA in muscles is higher than in the diets, which indicates that fish selectively retain this essential FA in their bodies (Panini et al., 2017). However, EPA content in fillets was lower than that found in diets. According to Sealey et al. (2011), the reasons for such differences are unclear. Other authors reported similar findings for rainbow trout (Belforti et al., 2015), blackspot sea bream (*Pagellus bogaraveo*) (Iaconisi et al., 2017), tilapia (Sánchez-Muros et al., 2017), and shrimp by including TM in the diet (Panini et al., 2017) and for rainbow trout when fishmeal was replaced by HI (Renna et al., 2017).

LA was the dominant FA in TM meal (37.5%), and this amount was similar to that obtained by Iaconisi et al. (2017) (36.4%) but higher than that observed by Cito et al. (2017)(20.6%). As expected, the LA percentage was higher in TM-fed fish, and a higher percentage was obtained

for T30-fed fish. These results agree with those reported by Belforti et al. (2015) and Iaconisi et al. (2017) in rainbow trout and blackspot sea bream, respectively, using TM larvae for partial substitution of FM. LA is usually transformed into arachidonic acid (ARA 20:4*n*-6), which influences several physiological functions, and ARA percentages are almost constant despite the increase of LA in diets.

In human diets, an adequate ratio between *n*-3 and *n*-6 PUFAs is necessary since competition for elongase and desaturase enzymes acting on both PUFA series is exercised. *n*-3 PUFAs promote anti-inflammatory effects, while most *n*-6 PUFAs contribute to inflammation (Rodríguez-Cruz et al., 2005). In fact, the *n*-3/*n*-6 ratio and the amounts of EPA and DHA are used as indicators of the health benefits of fish devoted to human consumption (Iaconisi et al., 2017).

Both insects (HI and TM) show a low *n*-3/*n*-6 ratio (Table 2), similar to previous findings (Nogales-Mérida et al., 2019). High LA amounts were found in TM meal, which induced a *n*-3/*n*-6 ratio up to 4 times lower than that of HI meal (Table 2). Since the three insect-fed fish experienced a significant reduction in the *n*-3/*n*-6 PUFA ratio, the quality of the fillets was adversely affected (Table 4). Similarly, other authors reported that the replacement of FM by TM resulted in reduced *n*-3/*n*-6 PUFA ratios: 0.41 in rainbow trout (Belforti et al., 2015), 0.50 in shrimp (Panini et al., 2017), 1.38 in blackspot sea bream (Iaconisi et al., 2017), and 0.74 in tilapia (Sánchez-Muros et al., 2017).

An important benefit of *n*-3 PUFAs is the prevention of coronary disease. For this reason, AI and TI indices were computed, which evaluate the possible benefits of the FA profiles on coronary diseases (Renna et al., 2017). In this study, only HI-fed fish had slightly increased AI in the three fish species, while TI only increased in sea bream and rainbow trout when they consumed insect feed. The results for TI were similar to those for rainbow trout (Belforti et al., 2015) and for blackspot sea bream (Iaconisi et al., 2017). For AI, our results are consistent with those of Iaconisi et al. (2017) but disagree with those of Belforti et al. (2015), who observed a decrease in AI when TM was included in feeds. However, the increase observed here was much lower than that obtained by Renna et al. (2017) in rainbow trout, who stated that values lower than 1.0 can be considered healthy for humans. In our worst result, with feed H30, we did not obtain a value higher than 0.5. These dissenting results could be due to different fish species, different diets and different composition of insect.

4.2. Comparison among species

When comparing the concentrations of the most physiologically important *n*-3 PUFAs in the fillets among the different fish species (Fig. 1), a different trend was noted between rainbow trout and the other two species. According to Sargent et al. (2003), freshwater and marine fishes show different requirements for *n*-3 and *n*-6 PUFAs. Marine fish can eat EPA- and DHA-rich prey; however, the prey of freshwater fish contain primarily ALA, low EPA content and very low amounts of DHA (Tocher, 2003). Therefore, freshwater fish fulfill their EPA and DHA requirements through increased elongation and desaturation activities, while marine fish, such as sea bream, lack the enzyme systems, or they have reduced activity (Mourente and Tocher, 1993, 1998; Ghioni et al., 1999). However, in our study, this was not been noted. In this way, EPA levels in rainbow trout and sea bream slightly decreased in insect-fed fish with respect to amounts in the control, although in tench this decrease was not significant. However, for DHA, a very different response was observed in rainbow trout. Although the level of DHA was higher in the control fish in the two freshwater species, tench and rainbow trout (Fig. 1c), the response when substituting with insect meal was very uneven. Unexpectedly, in rainbow trout, a freshwater species, the level of DHA in muscle decreases markedly, especially when fed with TM diets, while in tench or sea bream this decrease in DHA was not significant. It could be thought that rainbow trout fed insect meal, especially TM feed, consume DHA from the diet to cover some energy need, which neither tench nor sea bream have. Normally, cold water fish need higher amounts of *n*-3 PUFAs to maintain membrane fluidity (Molina-Poveda, 2016), but this would not explain why TM-fed rainbow trout use more DHA than control trout, since both have been raised at the same temperature. Thus, other unknown physiological mechanisms likely play a role.

Both salmon and rainbow trout have the ability to convert ALA into EPA and DHA (Henderson and Tocher, 1987; Tocher, 2003). HI and TM contains low ALA percentages (0.6% and 1.7% of total FA) (Table 2), which likely prevented rainbow trout from biosynthesizing DHA from ALA. However, ALA percentages increased slightly in TM-fed rainbow trout fillets, which have lower DHA amounts. This result is interesting, not only because of the biological/functional importance of DHA but also because it constitutes almost a quarter of all the FAs in the control trout (23%), although it decreased to half using T30 feed.

SFAs showed a similar response in the three fish species (Fig. 2a), with an increase in HI-fed fish, mainly due to the LaA content. For MUFAs (Fig. 2b) and PUFAs (Fig. 2c), differences among fish can be noted. In trout, and contrary to what occurred in tench and sea bream, MUFA levels increase markedly when using TM feeds. As TM meal is rich in OA, fish accumulate it in their fillets, specially in trout. For PUFAs the effect is inverse only for trout, starting from the higher proportion in the fillet of the control trout (45.0%) and decreasing for HI-diets, but the lowest levels were reached (36.5%) in TM-fed trout. This decline is a clear consequence of the drop in DHA indicated above.

As already stated, the replacement of FM by insect meal causes clear decreases in the *n*-3/*n*-6 ratios in the three fish species studied (Fig. 3a). The lowest levels were obtained in fish fed with TM diets, mainly due to the higher level of LA, but again, it was in trout where this decrease was more marked due to the higher decrease in *n*-3 PUFAs (mainly DHA) and also by the increase in *n*-6 (mainly LA). Foods with a high *n*-3/*n*-6 ratio have high nutritional value, and the progressive decrease in the *n*-3/*n*-6 ratio in the Western diet is cause for concern. Therefore, it is recommended that the optimal concentration for human health should vary between 0.25 and 1 (Simopoulos, 2002). Although the *n*-3/*n*-6 ratio decreased in the three fish species in this experiment, all fillets, including trout with the T30 feed (1.3), exceeded the minimum level of 1.0. In general, fish is considered a “healthy product”, and therefore, its consumption is recommended. According to our results, we can indicate that although the health value of lipid profiles of fish that consumed insects worsened in relation to the control fish, they still present a beneficial level of the *n*-3/*n*-6 PUFA ratio. In relation to other “quality” indices of the fillet, as noted in Fig. 3b, in the three fish species, there were similar increases in AI in HI-fed individuals (due to the high LaA amounts). In contrast, H30- and T30-fed gilthead breams and trout showed increases in AI (Fig. 3c) due to decreases in the *n*-3 PUFA content.

5. Conclusions

Based on the fillet FA profiles, the replacement of fish meal with insect meal was suitable for tench and, to a lesser extent, for sea bream, although concentrations of beneficial FAs tended to worsen with the inclusion of insect meal. However, for trout, due to its higher need for LCPUFAs (National Research Council, 2011), the inclusion of insects induces decreases in EPA, DHA, PUFAs, and the *n*-3/*n*-6 ratio, while IT causes increases. The FA profiles of HI and TM detected here differ from those referenced to some extent, due to differences in insect diets, larval stage at which the insect meal was obtained, and breeding conditions. Standardizing insect production systems to obtain homogeneous and constant feed is a priority. On the other hand, defatted insect meal could avoid undesirable effects by reducing the SFA content, as well as increase the level of high-quality protein. Similar effect might be obtained by previous enrichment of larvae with *n*-3 PUFAs, by means of using PUFAs-rich substrates in breeding systems, as previously reported by Barroso et al. (2017, 2019) and St-Hilaire et al. (2007).

Declaration of Competing Interest

None.

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CAPÍTULO III: *Facing the challenge of discarded fish: improving nutritional quality of two insect species larvae for use as feed and food*

Debido a las políticas de la editorial no poseo los derechos a presentar el trabajo en el formato publicado. A continuación, se adjunta el documento enviado a la revista Journal of Insects as Food and Feed sin los cambios sugeridos por los revisores. La versión final del artículo puede encontrarse con el siguiente DOI: 10.3920/JIFF2020.0019.

Due to the publisher's copyright policies, I do not have the rights to attach the paper in the published format. Attached below is the paper submitted to the Journal of Insects as Food and Feed without the changes suggested by the reviewers. The final version of the article can be found with the following DOI: 10.3920/JIFF202020.0019.

Facing the challenge of discarded fish: improving nutritional quality of two insect species larvae for use as feed and food

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Abstract:
Fishery discards represent 10% of total fishery catches, and insect rearing can be accomplished using this wasted resource. Considering that fish are the main source of n-3 very long-chain polyunsaturated fatty acids for human nutrition, and that fish contain both eicosapentaenoic acid and docosahexaenoic acid, this study focused on monitoring the accumulation of such n-3 very long-chain polyunsaturated fatty acids in insect larvae. To determine the feasibility of this process, we monitored nutritional changes achieved in two insect larvae—black soldier fly (*Hermetia illucens* Linnaeus, 1758) and mealworm (*Tenebrio molitor* Linnaeus, 1758) —fed using two different fish species from discards, i.e. round sardinella (*Sardinella* aurita Valenciennes, 1847) and blackspot seabream (*Pagellus bogaraveo*, Brünnich, 1768). Five different diets were prepared: control (broiler feed), 50% discarded fish (round sardinella and blackspot seabream)+50% broiler feed and 100% discarded fish. The 100% Blackspot seabream fed *H. illucens* accumulated eicosapentaenoic acid and docosahexaenoic acid up to2.4 g /100 g and 0.8 g /100 g, respectively. *T. molitor* accumulated lower amounts of both n-3 very long-chain polyunsaturated fatty acids due to the low intake of fish-containing feed by the larvae.

Keywords: *fatty acid, insect-meal, EPA, DHA, discarded fish*

The authors declare that there is no conflict of interest.

1. Introduction

The current world population of 7.7 billion is projected to reach 9.7 billion in 2050, and 11.2 billion in 2100. This continued fast growth demonstrates that new environmental challenges will be faced (UN, 2019), especially concerning food and feed production.

The population growth implies an increase in the demand for high-quality protein, which can be fulfilled by farm-animal products, although this option has a high environmental cost. To solve this issue, insects can be used as an alternative source of protein. They have advantages over traditional protein sources, including high feed conversion efficiency, lower ecological footprint, less impact on deforestation, and exceptional adaptation to being fed with food by-products (Oonincx 2017, van Huis 2013). Insect also has been described as a valuable protein source in animal nutrition, especially for fish, reducing environmental pressure of aquaculture, by decreasing the dependence of fish and soy meal to produce feed leading to obtain sustainable protein to cover aquaculture industry demand.

Aquaculture production, including aquatic plants, in 2016, was 110.2 MT with a value of 243.5 USD billion (FAO 2018). The aquaculture industry has grown faster than other major food sector, and is expected to grow 17% from 2016 to 2030, representing 45% of global fish production (FAO, 2018). Fishmeal is one of the main components of feed used in aquaculture, and approximately 20 MT from total fishery catches are derived for fishmeal and fish oil. Due to the high growth of aquaculture and future demands, the use of fishmeal will not meet the needs and makes the process more expensive (Ayadi et al., 2012). In this way, the replacement of fish meal with insect meal could help aquaculture achieve greater environmental sustainability. Different studies have shown that insects are a potential source of protein, making them a sustainable ingredient for animal diets (Barroso et al., 2014, Sanchez-Muros et al., 2014).

Recent studies have focused on insect meal as a suitable ingredient to include in fish diets (Bruni et al., 2018, Iaconisi et al., 2017). This is because most insect species have high amounts of protein (Sánchez-Muros et al., 2014), including adequate essential amino acids such as lysine, methionine, and leucine; i.e. those that are scarce in vegetal proteins (Tessari et al., 2016). For soy and fish meal, which constitute the most valuable protein source included in aquaculture feeds, some insect species display similar levels of protein as fish meal and increased content compared to soy meal; however, essential amino acid content is never superior to that found in fish meal (Sánchez-Muros et al., 2014).

The most abundant terrestrial n-3 long-chain polyunsaturated fatty acid (LCPUFA) is α-linolenic acid (ALA, 18:3n3). Insects fail to derivatize this PUFA to very-LCPFA (VLPUFA), i.e., eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3). Thus, for obtaining EPA- and DHA- rich insect meal both VLCPUFAs should be incorporated in adequate food sources for insects (Hixson et al., 2015). In animals, n-3 VLCPUFAs play a pivotal role in maintaining health, as they are involved in several metabolic pathways, membrane structure, eicosanoid production, and the control of lipid homeostasis (Punia et al., 2019). In humans, due to the low conversion rate achieved in the metabolic pathway for ALA conversion to EPA and DHA, these VLCPUFAs need to be present in dietary sources. However, as previously stated, terrestrial insects constitute a poor source of such VLCPUFAs, and this is a drawback for using them as feed. However, it has been previously revealed that the fatty acid (FA) profiles of insects can

be modified through dietary strategies (Kouba and Mourot 2011), which can be applied for large-scale insect rearing (Rutaro et al., 2018, Dreassi et al., 2017). For instance, increased n-3 VLCPUFAs content was achieved in black soldier fly (BSF, *Hermetia illucens*) larvae using fish meal or fish offal as feed ingredients (Barroso et al., 2017; Barroso et al., 2019).

Fishery discards represent approximately 10% of total fishery catches and are mainly derived from large-scale fishery industries (Zeller et al., 2016). Fishery discards are those fish that are thrown overboard due to different causes, mainly of economic, legal and technical origin (Tsagarakis et al., 2013). On 1 January 2019, the European Union (EU) launched new land rules for managing discarded fish (Art. 15, EU-CFP 2013). Such regulation, is intended to quantify the total volume of fishing catches while encouraging selectivity for fishing activity (Sardà et al. 2015). Compliance with this law is difficult in fishing ports because discarded fish generates a large amount of waste. Interestingly, this waste includes large quantities of n-3 VLCPUFAs, while discards are usually produced in ports lacking the infrastructure to produce fishmeal. Consequently, discarded fish becomes potentially unusable organic matter.

BSF larvae (BSFL) and mealworm (*Tenebrio molitor)* larvae (MWL) meals are becoming to be used as feed in aquaculture (Henry et al., 2015), and both larvae are considered promising candidates for industrial insect production (Veldkamp et al., 2012). BSF (Diptera) has a larval stage of 13 to 18 days, with the total life cycle is 30 to 40 days under optimal conditions. As a detrivorous species, can besuccessfully reared using a wide variety of organic waste. BSFL have crude protein and ether extract ranging from 32 to 48% and from 12 to 39% respectively (Wang and Shelomi 2016). MWL (Coleoptera), are considered a pest of grains and stored food (Ramos-Elorduy et al., 2002). This species has a highly variable life cycle, ranging from 280 to 630 days (Makkar et al. 2014). Its larval phase, which is composed of 9 to 12 stages, spans 3 to 4 months (Gullan and Cranston 2000). MWL contain crude protein (CP) and ether extract ranging from 47 to 53% and from 33 to 43% respectively (Oonincx et al., 2015, Finke 2015, Bernard et al., 1997). Mineral composition is similar in BSF and MWL, with higher Ca, Fe and Mn in BSFL and higher Na in MWL (Janssen et al., 2019)

Previous studies have focused on fish waste and discarded fish to improve the n-3 VLCPUFAs content of BSFL (Barroso et al., 2019, St-Hilaire et al., 2007). Such research demonstrated that fish-fed BSFL accumulate n-3 VLCPUFAs and that such accumulation increases as larvae are fed with discarded fish for longer durations. In this way, producers should decide whether they prefer a high n-3 VLCPUFAs increase in larvae with more complex management in BSFL breeding or to simplify management and discomfort while although obtaining lower n-3 VLCPUFAs enrichment in larvae (Barroso et al., 2019). An important issue to be elucidated is whether suitable amounts of n-3 VLCPUFAs can be obtained in BSFL through partial substitution of insect feed with discarded fish, or whether the use of fish derivatives as single feed component is required for such achievements. For MWL there is no information on this subject. An additional problem is that discarded fish contain highly variable and species-dependent n-3 VLCPUFAs amounts, thus leading to erratic n-3 VLCPUFAs enrichment. Moreover, knowledge about the ability of these larvae to consume discarded fish is lacking, as well as certainty about the reproducibility obtained by different trials concerning n-3 VLCPUFAs content. In short, this work evaluates the n-3 VLCPUFAs enrichment obtained in two insect larvae—*H. illucens* and *T. molitor*.

2. Materials and methods

2.1.Experimental design

BSFL and MWL were reared at the laboratories of University of Almería. The experiment was performed at 26±1°C and 65±5% humidity. Commercial broiler feed (NANTA®, Spain) was used as control feed (60% feed and 40% distilled water w/v for BSFL and 95% feed and 5% distilled water w/v for MWL). All larvae were fed the control diet for 3 days. Three-day post hatch larvae were used. After this period of acclimation, the larvae were divided into groups of 3,000 larvae separated into three 15 x 20 cm boxes, resulting in a larval density of 3.33 larvae cm^2. For this experiment, two different species of discarded fish were tested—round sardinella (RS, *Sardinella aurita*) and blackspot seabream (BS, *Pagellus bogaraveo*) — and were captured in the Mediterranean bay of Almería (Spain). RS has limited gastronomic value because it contains many fine bones, while the BS were not of the regulated commercial size.

The experimental diets were formulated by replacing 0%, 50% and 100% of commercial broiler feed (NANTA®, Spain) with RS or BS. A total of 6 different diets were assessed: C (control, chicken broiler feed), RS50, RS100, BS50 and BS100 (50 and 100 correspond to percentage of chicken broiler feed substituted by dried discarded fish). Insects were fed ad libitum. For produce the feed, fish heads were removed, and the remaining fish body was cut into 4-cm slices. BSFL were reared using fresh fish; for MWL fish was dried before using (45-50 ºC, 2 days). BSFL was fed for 7 days due to its high voracity and short life cycle, while MWL were fed for 15 days.

The gross composition of diets and fish discard as well as the FA profiles are shown in Table 1.

Table 1. Gross composition and fatty acid profiles (FA% of total FA) of ingredients of experimental diets

	Control	RS	BS
Gross composition g/100 g dry wt			
Crude protein	21.6	79.4	66.2
Ether extract	4.6	7.	15.5
Ash	5.9	11.4	13.2
Fatty acids (FA% of total FA)			
14:0	n.d.	4.5	3.5
16:0	15.7	24.1	20.6
16:1*n*7	n.d.	2.8	4.6
18:0	2.7	7.5	7.7
18:1*n*9	24.0	8.2	15.7
18:1*n*7	n.d.	2.3	4.9
18:2*n6*	53.7	1.8	1.7
18:3*n*3	3.9	n.d.	0.9
18:4*n*3	n.d.	n.d.	0.9
20:1*n*9	n.d.	1.9	1.0
20:4n6	n.d.	2.2	2.7
20:5*n*3	n.d.	9.1	11.0
22:5*n*3	n.d.	1.7	3.7
22:6*n*3	n.d.	33.2	15.8
SFA	18.4	38.8	36.4
MUFA	24.0	15.1	26.2
PUFA	57.6	48.1	36.8
n-3	3.9	44.0	32.4
n-6	53.7	4.1	4.4
20:5*n*3 (g/100 g DM)	n.d.	2.4	3.5
22:6*n*3 (g/100 g DM)	n.d.	0.7	2.6

RS: *Sardinella aurita*; BS: *Pagellus bogaraveo*; SFA: saturated fatty acid; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

2.2.Samples

BSFL and MWL were collected before the pre-pupal stage (at approximately 7 and 15 days, respectively) and 24 h after feeding. Larvae were slaughtered and stored at -20 ºC prior to being lyophilized in a freeze-dryer. Complete body of larvae were ground and homogenized.

2.3. FA analyses

FA analyses were carried out as in previous studies (Lepage and Roy, 1984; Rodríguez-Ruiz et al., 1998). FA was measured after direct derivatization to FA methyl esters (FAME). For this, 50 mg of freeze-dried sample was weighed and placed in test tube. Then 0.5 mg of internal standard (nonadecanoic acid, 19:0), 2 mL of a methylating mixture (methanol:acetyl chloride 20:1 v/v) and 1 mL of *n*-hexane were added. The tubes were then capped and heated at 100 ºC for 30 min. After the tubes were cooled to room temperature, 1 mL of distilled water was added, and then the tubes were centrifuged (2000 × g, 5 min). The organic layer was collected for GC-FID analysis. FAME were analysed in a Focus GC (Thermo Electron. Cambridge. UK) equipped with a flame ionisation detector (FID) and an Omegawax 250 capillary column (30 m x 0.25 mm i.d. x 0.25 μm film thickness; Supelco. Bellefonte. USA), as previously described (Guil-Guerrero et al., 2014). The peak area of the internal standard was used as a reference to calculate the mass of each FA in the resulting chromatograms, and the results were computed as FA% of total FA. The relative retention factors for each FA as reported by (Cladis et al., 2014) were considered for quantification.

Quality control of FA analyses was performed according to previous protocols (Barroso et al., 2017, Griffiths et al., 2010, Guil- Guerrero et al., 2013).

2.4. Gross composition

The nutritional profile of BSFL, MWL and fish used in all experiments was analysed according to the standard procedures of the Association of Official Analytical Chemists (AOAC, 2005), with specific methods as follows: CP content was determined by Kjeldahl * 6.25 (AOAC, 2005; #954.01) (Nx6.25), crude fat (EE) was determined by ethyl ether extraction (Soxhlet technique) (AOAC, 2005; #920.39), the moisture was gravimetrically quantified by drying at 105±0.5 °C (AOAC, 2005; #934.01), while ash was gravimetrically determined after combustion at 500 °C in a muffle furnace (AOAC, 2005; #942.05) to a constant weight. All analyses were performed in triplicate.

2.5. Statistical analyses

The models were fitted by a maximum quasi-likelihood estimation with the GenLin procedure with gamma errors and the Logarithm link function using the IBM SPSS version 25.0 statistical software package (IBM, 2015). In each trial, the significance of the model was assessed by an Omnibus test. For each regression effect specified in the model, a Wald statistic was conducted. This is a test based on linearly independent pairwise comparisons among the estimated marginal means. The mean values were then compared pairwise with significance a level of $P < 0.05$ using the statistical software IBM SPSS statistics 25 (IBM, 2015).

3. Results

3.1. Gross composition

The gross composition of BSFL and MWL feed with different concentrations of discarded fish are detailed in Tables 2 and 3, respectively.

Table 2. Gross composition (% dry wt) of *Hermetia illucens* fed with experimental diets

	BSFL-C			BSFL-RS50			BSFL-RS100			BSFL-BS50			BSFL-BS100		
Ether extract	28.1	±	0.7^{d}	30.4	±	0.7^{c}	31.7	±	0.8^{bc}	28.4	±	0.7^{bd}	34.2	±	0.8^{a}
Crude protein	44.8	±	0.4^{c}	51.3	±	0.5^{a}	55.4	±	0.6^{d}	50.3	±	0.4^{ab}	50.4	±	0.4^{ab}
Ashes	8.2	±	0.1^{b}	6.2	±	0.1^{c}	5.2	±	0.1^{a}	5.9	±	0.0^{d}	5.3	±	0.0^{a}

Means (± SE) from 3 individual measurements. the same letters within a row are not significantly different from one another (chi2 of Wald. P < 0.05). BSFL-C: black soldier fly larvae fed with broiler feed; BSFL-RS50: black soldier fly larvae fed with 50% broiler feed and 50% sliced Sardinella aurita. BSFL-RS100: black soldier fly larvae fed with sliced Sardinella aurita; BSFL-BS50: black soldier fly larvae fed with 50% control broiler feed and 50% sliced Pagellus bogaraveo. BSFL-RS100: black soldier fly larvae fed with sliced Pagellus bogaraveo.

Table 3. Gross composition (g/100 g dry wt) of *Tenebrio molitor* fed with experimental diets

	MWL-C			MWL-RS50			MWL-RS100			MWL-BS50			MWL-BS100		
Ether extract	34.7	±	0.9^{a}	32.5	±	0.9^{ab}	23.7	±	0.7^{b}	31.8	±	0.9^{b}	24.9	±	0.7^{b}
Crude protein	50.1	±	0.5^{b}	55.0	±	0.6^{a}	67.7	±	0.7^{c}	54.4	±	0.6^{a}	65.4	±	0.7^{d}
Ashes	3.3	±	0.0^{b}	3.5	±	0.1^{a}	4.2	±	0.1^{c}	3.6	±	0.0^{a}	4.9	±	0.0^{d}

Means (± SE) from 3 individual measurements. the same letters within a row are not significantly different from one another (chi^2 of Wald. P < 0.05). MWL-C: mealworm larvae fed with broiler feed; MWL-RS50%: mealworm larvae fed with 50% broiler feed and 50% sliced *Sardinella aurita*. MWL-RS100: mealworm larvae feeding with sliced *Sardinella aurita*; MWL-BS50: mealworm larvae fed with 50% broiler feed and 50% sliced *Pagellus bogaraveo*. MWL-BS100: mealworm larvae fed with sliced *Pagellus bogaraveo*.

BSFL increased lipids from 28 g in broiler-feed larvae to 30-34 g/100 g dry weight (wt) in BSFL-fed with discarded fish, with a significant difference between BS- and RS-100-fed larvae. For MWL, lipid content decreased in the 5 experimental diets compared with the MWL-feed control diet, and it was statistically significant with respect to larvae fed using both discarded fish at 100%.

CP increased significantly in all BSFL and MWL conditions compared to control larvae. The best results were obtained for 100% fish-fed larvae (RS100 and BS 100). BSFL-RS100 and BSFL-BS100 contained 55.4 and 50.4 g/100 g dry wt CP, respectively, while BSFL-C contained 44.8 g/100 g dry wt. Similarly, an increase in CP was obtained for MWL, varying from 50.1 in MWL-C to 67.8 in MWL-RS100 and 65.4 g/100 g dry wt in MWL-BS100.

3.2. Fatty acid composition

FA profiles of BSFL and MWL are summarized in Tables 4 and 5, respectively. Both BSFL and MWL FA profiles were related to the amounts of discarded fish used as feed components. Notice that saturated FA (SFA) decreased when discarded fish percentages increased for all BSFL cases, which was mainly related to a decreasing in lauric acid (LA, 12:0) content. In MWL, SFA content remained roughly the samein all experiments, and SFA content only increased for RS100-fed MWL. For BSFL, monounsaturated FA (MUFA) amounts increased in discarded fish-fed larvae groups, reaching maximum MUFA content for BSFL-RS100 and BSFL-BS100, with 18.1 and 25.2% of total FA, respectively. Such increases were mainly due to the occurrence of palmitoleic acid (POA, 16:1n7) in discarded fish-fed larvae and oleic acid (OA, 18:1n9). For MWL, MUFA content decreased in 100%-discarded fish-fed larvae, and this decline mainly affected OA. For BSFL, PUFA percentages remained at similar levels in almost all discarded fish-fed larvae, although minor reduction were noted for RS50-fed BSFL (11.7% of total FA) while the levels increased for BS100-fed BSFL(15.1% of total FA). PUFA content in MWL increased for discarded fish-fed larvae, although with no great differences in

comparison to control-fed MWL. PUFA remained unchanged or decreased for discarded fish-fed BSFL. n-3 VLCPUFAs reached maximum percentages in RS100-fed BSFL and BS100-fed BSFL at 8.8 and 11.8% of total FA, respectively, when compared with broiler-diets fed larvae.

Table 4. Fatty acid profiles (FA% of total FA) of *Hermetia illucens* fed with experimental diets

	BSFL-C			BSFL-RS50			BSFL-RS100			BSFL-PB50			BSFL-PB100		
10:0	1.3	±	0.1^{a}	1.1	±	0^{a}	1.0	±	0.1^{b}	1.0	±	0.0^{b}	1.1	±	0.1^{ab}
12:0	45.7	±	0.8^{a}	42.4	±	0.7^{b}	39.0	±	0.8^{c}	35.3	±	0.6^{d}	28.1	±	0.5^{e}
14:0	8.9	±	0.2^{a}	8.7	±	0.2^{a}	8.3	±	0.2^{b}	7.8	±	0.1^{b}	6.7	±	0.1^{c}
16:0	12.9	±	0.3^{b}	15.5	±	0.3^{a}	17.8	±	0.4^{c}	15.6	±	0.3^{a}	16.7	±	0.4^{d}
16:1n7	2.1	±	0.1^{a}	3.1	±	0.1^{b}	4.2	±	0.2^{c}	5.6	±	0.3^{d}	7.9	±	0.5^{e}
18:0	2.7	±	0.1^{c}	2.7	±	0.1^{c}	3.1	±	0.1^{b}	3.4	±	0.1^{a}	3.2	±	0.1^{ab}
18:1n9	10.3	±	0.4^{c}	11.6	±	0.3^{b}	13.9	±	0.5^{a}	14.2	±	0.4^{a}	15.2	±	0.5^{a}
18:1n7	n.d.^{a}			n.d.^{a}			n.d.^{a}			1.7	±	0.0^{b}	2.1	±	0.1^{c}
18:2n6	12.1	±	0.4^{a}	6.6	±	0.2^{b}	4.2	±	0.1^{c}	4.3	±	0.1^{c}	3.3	±	0.1^{d}
18:3n3	1.0	±	0.0^{a}	0.8	±	0^{b}	1.1	±	0^{a}	0.6	±	0.0^{c}	0.8	±	0.0^{b}
18:4n3	1.5	±	0.1^{a}	1.12	±	0.1^{b}	0.9	±	0.1^{c}	1.2	±	0.1^{ab}	1.2	±	0.1^{b}
20:1n9	n.d.^{a}			0.7	±	0^{b}	n.d.^{a}			n.d.^{a}			n.d.^{a}		
20:4n6	n.d.^{a}			n.d.^{a}			1.2	±	0.1^{b}	0.9	±	0.0^{c}	1.1	±	0.1^{b}
20:5n3	n.d.^{a}			2.8	±	0.1^{b}	4.2	±	0.3^{c}	5.1	±	0.3^{d}	7.1	±	0.4^{e}
22:6n3	n.d.^{b}			1.4	±	0.1^{c}	2.6	±	0.1^{a}	2.2	±	0.1^{a}	2.6	±	0.1^{d}
SFA	71.6	±	0.6^{a}	70.4	±	0.5^{a}	69.2	±	0.6^{b}	63.2	±	0.4^{c}	55.8	±	0.5^{d}
MUFA	12.42	±	0.5^{a}	15.4	±	0.5^{b}	18.1	±	0.7^{c}	21.5	±	0.7^{d}	25.2	±	0.9^{e}
PUFA	13.1	±	0.4^{b}	11.7	±	0.3^{c}	13.2	±	0.4^{b}	13.1	±	0.3^{b}	15.1	±	0.4^{a}
n-3 PUFA	2.51	±	0.1^{d}	6.3	±	0.3^{c}	8.8	±	0.5^{b}	9.1	±	0.4^{b}	11.8	±	0.6^{a}
n-6 PUFA	10.58	±	0.4^{a}	5.4	±	0.2^{b}	4.5	±	0.2^{c}	3.9	±	0.1^{d}	3.3	±	0.1^{e}

Means (± SE) from 3 individual measurements. with the same letters within a row are not significantly different from one another (chi^{2} of Wald. P < 0.05). SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids. BSFL-C: black soldier fly larvae fed with broiler feed; BSFL-RS50: black soldier fly larvae fed with 50% broiler feed and 50% sliced Sardinella aurita. BSFL-RS100: black soldier fly larvae fed with sliced Sardinella aurita; BSFL-BS50: black soldier fly larvae fed with 50% control broiler fed and 50% sliced Pagellus bogaraveo. BSFL-BS100: black soldier fly larvae fed with sliced Pagellus bogaraveo. nd: not detected (Limit of Detection (LOD) ≈ 0.02 mg/100 g FW).

Table 5. Fatty acid profiles (FA% of total FA) of *Tenebrio molitor* fed with experimental diets

	MWL-C			MWL-RS50			MWL-RS100			MWL-PB50			MWL-PB100		
10:0	n.d.			n.d.			n.d.			n.d.			n.d.		
12:0	0.4	±	0.0	0.3	±	0.0	n.d.			0.3	±	0.0	n.d.		
14:0	3.6	±	0.0^{b}	3.6	±	0.0^{b}	3.8	±	0.0^{a}	3.6	±	0.0^{b}	3.5	±	0.0^{c}
16:0	15.1	±	0.1^{b}	15.1	±	0.1^{b}	16.5	±	0.1^{a}	15.1	±	0.1^{b}	14.6	±	0.1^{c}
16:1n7	1.2	±	0.0^{a}	1.0	±	0.0^{b}	3.6	±	0.0^{c}	0.9	±	0.0^{d}	4.2	±	0.0^{e}
18:0	3.0	±	0.0^{c}	3.1	±	0.0^{bc}	3.9	±	0.1^{a}	3.2	±	0.0^{b}	3.9	±	0.1^{a}
18:1n9	47.5	±	0.2^{a}	47.0	±	0.2^{a}	39.6	±	0.2^{b}	47.2	±	0.2^{a}	38.7	±	0.2^{c}
18:1n7	n.d.^{a}			n.d.^{a}			0.5	±	0.0^{c}	n.d.^{a}			2.3	±	0.0^{b}
18:2n6	25.4	±	0.1^{a}	25.2	±	0.1^{a}	21.2	±	0.1^{b}	24.7	±	0.1^{c}	22.2	±	0.1^{d}
18:3n3	0.8	±	0.0^{a}	0.9	±	0.0^{b}	1.3	±	0.0^{c}	0.9	±	0.0^{d}	1.1	±	0.0^{e}
18:4n3	n.d.^{a}			n.d.^{a}			0.7	±	0.0^{b}	n.d.^{a}			0.6	±	0.0^{c}
20:1n9	n.d.^{a}			n.d.^{a}			1.2	±	0.0^{b}	n.d.^{a}			n.d.^{a}		
20:4n6	n.d.^{a}			n.d.^{a}	±		0.7	±	0.0^{b}	0.3	±	0.0^{c}	1.1	±	0.0^{d}
20:5n3	n.d.^{a}			0.6	±	0.0^{b}	3.3	±	0.0^{c}	0.8	±	0.0^{d}	3.6	±	0.0^{e}
22:5*n*3	n.d.^{a}			n.d.^{a}			n.d.^{a}			n.d.^{a}			0.4	±	0.0^{b}
22:6n3	n.d.^{a}			n.d.^{a}			1.5	±	0.0^{b}	n.d.^{a}			1.3	±	0.0^{c}
SFA	22.1	±	0.1^{b}	22.1	±	0.1^{b}	24.1	±	0.1^{a}	22.2	±	0.1^{b}	21.9	±	0.1^{b}
MUFA	48.7	±	0.2^{a}	48.1	±	0.2^{b}	44.9	±	0.2^{c}	48.2	±	0.2^{ab}	45.2	±	0.2^{c}
PUFA	26.2	±	0.1^{a}	26.1	±	0.1^{a}	24.8	±	0.1^{b}	25.9	±	0.1^{c}	26.1	±	0.1^{ac}
n-3 PUFA	0.8	±	0.0^{a}	1.5	±	0.0^{b}	6.8	±	0.0^{c}	1.7	±	0.0^{d}	7.0	±	0.0^{e}
n-6 PUFA	25.4	±	0.1^{a}	24.6	±	0.1^{b}	18.0	±	0.1^{c}	24.2	±	0.1^{d}	19.1	±	0.1^{e}

Means (± SE) from 3 individual measurements. the same letters within a row are not significantly different from one another (chi^{2} of Wald. P < 0.05. SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids. MWL-C: mealworm larvae fed with broiler feed; MWL-RS50: mealworm larvae fed with 50% broiler feed and 50% sliced *Sardinella aurita*. MWL-RS100: mealworm larvae feeding with sliced *Sardinella aurita*; MWL-BS50: mealworm larvae fed with 50% broiler feed and 50% sliced *Pagellus bogaraveo*. MWL-BS100: mealworm larvae fed with sliced *Pagellus bogaraveo*. nd: not detected (Limit of Detection (LOD) ≈ 0.02 mg/100 g FW).

Considering MWL, n-3 PUFA also improved for discarded fish-fed larvae, reaching maximum percentages for RS100-fed MWL- and BS100-fed MWL at 6.8 and 7.0% of total FA, respectively. Such n-3 PUFA improvement was mainly due to an increase of

both EPA and DHA. Figure 1 shows both EPA and DHA amounts (g/100 g dry wt) contained in experimental diet-fed BSFL and MWL.

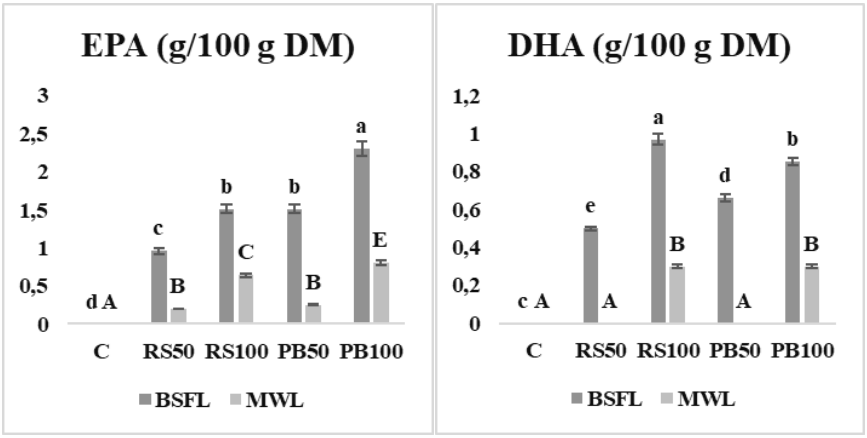

Figure 1. EPA and DHA (g/100 g DM) found in BSFL and MWL insect meal. Means (± SE) from 3 individual measurements. the same letter within a column indicates not significantly different from one another (chi^2 of Wald. $P < 0.05$).

Notice that EPA+DHA increased to 3.15 g/100 g dry wt in BS100-fed BSFL, and this amount was mainly due to EPA, which reached 2.30 g/100 g dry wt of insect meal. BSFL showed a similar trend: EPA+DHA percentages varied from 2.17 in BS50 to 2.48 g/100 g dry wt in RS100), and this was related to the high EPA+DHA percentage contained in BS (2.42 and 3.48 g/100 g/100 g dry wt of EPA and DHA, respectively). EPA and DHA amounts in MWL increased when discarded fish were added, although such improvement was lower than that obtained for BSFL. When both discarded fish reached 50% feed in the MWL diet, DHA was not detected, while EPA reached 0.20 and 0.25 g/100 g dry wt for RS50- and BS50-fed MWL, respectively. For BS- and RS-100 fed MWL, DHA amounts increased to 0.30 g/100 g dry wt, while EPA increased to 0.64 and 0.81 g/100 g dry wt in RS100- and BS100-fed MWL, respectively.

4. Discussion

Previous works demonstrated that the nutritional value of insects can be changed by means of dietary modifications. Such modification focuses on the gross composition of insects, especially on protein and fat content, in order to obtain a feed having a high nutritional value for either livestock, aquaculture or humans (Barroso et al., 2019, Patel et al., 2019, Pieterse et al., 2019). In this work, experiments were designed to achieve both modification of the gross composition and FA profiles of two insect larvae. This study was designed to focus on the increase of n-3 VLCPUFA in MWL and the effects obtained by using different fish species as feed components for BSFL; RS-fed BFSL were investigated in previous works (Barroso et al., 2019).

4.1 Gross composition of BSFL and MWL

For fish-fed larvae, the larval body showed significant differences in lipid percentages. For the two discarded fish species, in 100% fish-fed BSFL lipids content significantly increased; however, for 100% fish-fed MWL, lipids significantly decreased. Differences in lipids content could be due to different feeding behaviour of these two species: although MWL are omnivorous insects (Rho et al., 2014), which are typically fed on cereal bran or flour (wheat, oats, maize) (Ramos-Elorduy et al., 2002), whereas BSFL are reported as diverse organic material consumer (Wang and Shelomi, 2017). According to Li et al., 2016, MWL need at least 5% crude fiber in their diet in order to grow adequately,

although larval intake was not measured in this work. However, considering an absence of fibre in discarded fish, MWL likely lacked this compound for proper growth. In 50% fish-fed MWL (RS50 and BS50), total lipids decreased although lacking significant differences with respect to the amounts measured in control larvae, possibly because their diets contained 50% of the control feed, and they were fed *ad libitum*, which allows them to maintain fatty tissues. For BSFL, both fat and ash amounts seem to be dependent on the rearing substrate. In fact, BSFL reared on energy-dense substrates (low crude fibre amounts) turn into high-fat prepupae (Spranghers et al., 2017).

CP content in both BSFL and MWL increased when discarded fish were added to their diets, which can be due to the higher CP content present in discarded fish when compared to the control diet. CP amounts obtained for control diet-fed BSFL was similar to that obtained by Barroso et al., (2017) using fishmeal, and lower than other amounts obtained for discarded fish-fed BSFL (Barroso et al., 2019). Although CP increased for both BSFL and MWL, lipid amounts did not follow the same trend for both species: whereas for BSFL fat content increased when discarded fish was added to the feed, for MWL this nutrient decreased. CP increased in MWL when discarded fish was added to feed, which was mainly due to a decreased feed intake given that this induces a decline in the fat reservoir of the larvae, as well as to an increase of the percentage of the exoskeleton with respect to the total body weight. MWL intake was lower for 100% fish diets, which induced to a decreased lipid reservoir, although the remaining lipids have higher nutritional value than control diet-fed MWL, due to both lower SFA and higher n-3 VLCPUFA amounts, especially those corresponding to EPA and DHA, when compared to control diet-fed MWL. Therefore, the appropriate way to fed MWL to increase fish waste consumption is worthy of investigation, given that such larvae can bioaccumulate large amounts of n-3 VLCPUFA. Overall, FA profiles have been modified for both BSFL and MWL when any rich n-3 VLCPUFA source was used as feed ingredient, and similar to what was achieved in previous studies (Barroso et al., 2019, Lehtovaara et al., 2017, Rutaro et al., 2018).

4.2 *EPA and DHA accumulation in larvae*

In the present work, noticeable amounts of EPA and DHA were achieved in the BSFL body; however, for MWL such improvement was lower than that obtained for the previous larvae. This bioaccumulation was noted in parallel with the amounts of discarded fish used as feed in all trials.

Regarding n-3 VLCPUFA, EPA is well assimilated and stored in BSFL. As shown in Figure 1, BS-fed BSFL reached similar EPA amounts on dry wt (only 5% difference) with respect to that found in BS. However, BSFL do not accumulate DHA as efficiently as EPA, and the maximum DHA amount was achieved for BS100-fed BSFL (0.85 g/100 g dry wt). Such figures are higher than those obtained in a previous study using discarded round sardinella-fed BSFL at different times before slaughtering (Barroso et al., 2019). In this work, 12-day RS-fed BSFL reached EPA+DHA of 0.757 g/100g dry wt, while RS100-fed BSFL reached EPA+DHA at 2.48 g/100 g dry wt. This can be because that lipid percentages in BSFL differ in both experiments; in the present trial, RS-fed larvae contained lipids at ~30 g/100 g dry wt, while in the previous study, lipids reached 18 g/100 g dry wt for 12 days-fed larvae. Although both experiments were conducted for BSFL, in previous work, BSFL at different larval stages were used. The present study focused on the stage before pupation, in which lipids reached the highest amounts among all larval stages. In this regard, larvae store lipids for starvation and metamorphosis, and the lipids accumulated in larval stages are important for oogenesis processes (Arrese et al., 2010)

EPA and DHA amounts in fish-fed MWL were lower than those obtained for BSFL, and this was probably due to the ability for self-selection of diets described for MWL (Morales-Ramos et al., 2011); when control-broiler feed was available, such larvae preferably ingested this over discarded fish. This probably caused the decreased lipid amounts obtained when increasing the discarded fish percentages in the larval diet, which induces a fasting state.
This subject need further studies, as it may have implications for n-3 VLCPUFA-rich MWL obtainment. It has been demonstrated that MWL can accumulate n-3 VLCPUFAs, although the feeding behaviour of this species complicates fish use in feed, unlike for BSFL. Regarding experimental observations in this trial, fish intake was higher than that previously described. It is likely that, different ways of preparing discarded fish feed for MWL rearing will result in improved amounts of n-3 VLCPUFAs in the larval body.
It should be noted that different fish species influence both EPA and DHA content in the BSFL body, and more of lipids was noted in BS-fed larvae. However, this difference was not observed for MWL, as for both fish species, MWL displayed similar EPA and DHA amounts. This should be related to the lower MWL intake of fish, as well as for the selectivity noted for broiler feed intake with respect to intakes observed for 50% fish-feeds.
Both fish-fed BSFL and MWL display appropriate EPA+DHA amounts for use in aquaculture systems (Yildiz et al., 2008). EPA amounts in 50 and 100% BS- and RS-fed BSFL are within the normal range of fish feed (Yildiz et al., 2008), which were 0.8-1.8% of total FA, and DHA% was 1-3% of total FA. However, insect meal obtained in this experiment could be used as a partial substitute for fishmeal, and as a suitable ingredient for aquaculture feeds to increase DHA%.

4.3 Insects as resource of n-3 VLCPUFA

Both MWL and BSFL meal derived from fish discard-fed insects constitute a new source of n-3 VLCPUFA for humans, considering that n-3 VLCPUFA requirements for adults are approximately 250 mg daily (Sioen et al., 2017). Figure 2 shows changes in the n-6/n-3 ratio in BSFL and MWL. This ratio is a good indicator of the quality of the FA intake. A low n-6/n-3 ratio acts as a suppressor of occurrence of several diseases, while a high ratio can promote the onset of cancer and cardiovascular and inflammatory diseases, among others (Simopoulos 2016). Notice that the use of discarded fish in the diet of BSFL and MWL leads to an important decrease of this ratio.
Overall, insects are emerging as an interesting food resource for reducing the environmental impact of obtaining both human and animal foods. This growing interest in mass breeding of insects is based on their nutritional quality and the low environmental impact of their rearing.
The results obtained in this work indicate that BSL accumulates high amounts of n-3 VLCPUFAs. Given that some insects constitute a potential source of redox ingredients, insects could play a pivotal role as functional foods (Di Mattia et al., 2019). For the aversion new consumers might have to such animals, their acceptance could be increased according to presentations and mixes with other common foods (Kusch et al., 2019).
The use of insects used as “bio-accumulators” of high-quality protein and lipids constitutes an environment friendly way to seize discarded fish to follow new legislations. The EU allows the use of insects for food and feed (Article 3.6 of Regulation (EC) No 1069/2009). However, feeding larvae of insects with unprocessed animal protein, such as discarded fish, is still not allowed. It is expected that new studies and safety trials would enable a new legislative framework for solving this.

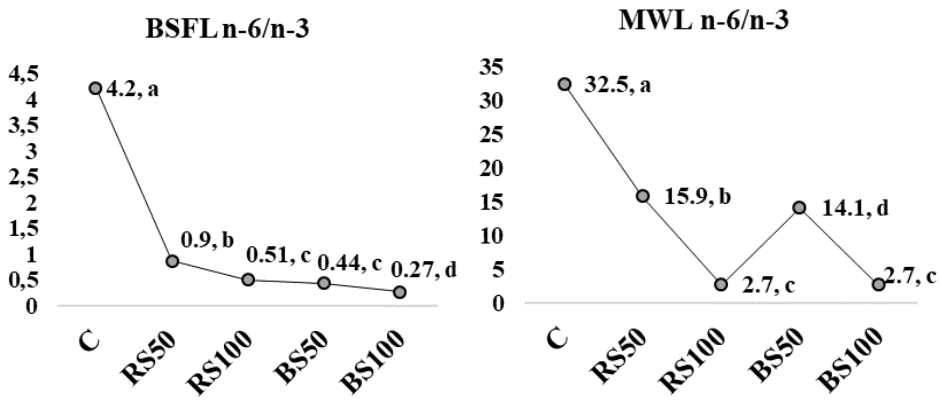

Figure 2. n-6/n-3 ratio of fish-fed BSFL and MWL. Means (± SE) from 3 individual measurements. the same letter within a column is not significantly different from one another (chi^2 of Wald. $P < 0.05$).

5. Conclusions

In this study, an alternative use for discarded fish as a component of insect diets has been shown. This is a suitable option when the use of discarded fish for fishmeal production is unattainable, due to a lack of infrastructure or economic underperformance. As demonstrated here, BSFL are the most appropriate insects to transform discarded fish into animal protein as well as to bioaccumulate large amounts of n-3 VLCPUFA, especially EPA. However, MWL are not as good as BSFL at managing such residues, as they are unable to consume large pieces of fish, and thus research about alternative fish preparations to increase MWL consumption is warranted. BSFL accumulated both EPA and DHA, which are not naturally present in insect larvae. The bioaccumulation of both VLCPUFA differs according to the larval species tested. Although current European Union legislation does not allow feeding larvae using animal sources, this study opens new perspectives for future revisions on this subject.

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CAPÍTULO IV: *Effect on Intermediary Metabolism and Digestive Parameters of the High Substitution of Fishmeal with Insect Meal in* Sparus aurata *Feed*

insects

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Article

Effect on Intermediary Metabolism and Digestive Parameters of the High Substitution of Fishmeal with Insect Meal in *Sparus aurata* Feed

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Simple Summary: The depletion of traditional protein sources and the impact this causes on the production costs of aquaculture feed make it necessary to find alternative materials that allow for the sustainability of production. Among various proposals, insects have drawn scholarly attention because of their high protein content and the efficiency of their production, both from an environmental and an economic perspective. However, nutritional changes in fish diets require further clarification regarding the effect of this new ingredient in fish performance and physiology. In this study, we evaluated the use of two insect meal species, *Hermetia illucens* and *Tenebrio molitor*, for the partial replacement of fishmeal, as well as their influence on growth indices and the gut microbiome. Although the results showed a worsening of biometric parameters and a modification of the microbial community, the impact was different depending on the insect species and their rearing conditions. Thus, specific studies for each case are recommended.

Abstract: *Hermetia illucens* and *Tenebrio molitor* were tested on account of their potential to replace fish protein in feed. Two levels of replacement for *H. illucens*, 30% and 50% (H30 and H50), and one for *T. molitor*, 50% (T50), as well as an additional diet with a modified fatty acid fraction (H50M), were investigated in relation to juvenile *Sparus aurata* growth indices, enzyme activities and gut microbiome. A T50 diet showed similar results to a control (C) diet, with no significant differences regarding morphological indices and minor differences for nutritional indices. Regarding the gut microbiome, H50M was the diet which showed the more similar prokaryotic community to C, which suggests that fatty acid fractions might influence the composition of the gut microbiome. Nevertheless, differences appeared to be related to a redistribution of dominant species, while changes in species affiliation were limited to minoritary species. The positive correlation between some of these minoritary species (*Peptostreptococcus russellii*, *Streptococcus dysgalactiae* and *Weisella confusa*) and several fish growth parameters might explain differences between control and insect diets. Deciphering such uncertainty and revealing the potential role these unusual species may play on fish performance should be addressed in future investigations.

Keywords: aquafeed; *Sparus aurata*; insect meal; *Tenebrio molitor*; *Hermetia illucens*; biometric parameters; gut microbiome

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1. Introduction

A circular economy has become a target objective in the movement towards sustainability. Insects play an important role in this change because their rearing has a small ecological footprint; less land and water is required, they emit fewer greenhouse gases, and they have a high feed conversion efficiency in comparison to traditional animal products. These factors should be considered in tandem with insects' capacity to transform low-value organic by-products into high-quality food or feed that can be used as animal feed or aqua feed [1].

The utilization of insect meal as a substitute in fishmeal has been studied in several fish species and insect species. High percentages of substitution have been used for feeding several species without compromising their growth ranges, as in the case of *Salmo salar* [2], yellow catfish [3] and Nile tilapia [4], with substitution levels of 100%, 75% and 68%, respectively. However, most of the studies reported 25% as the threshold at which growth indices can be negatively affected [5]. Among the most studied species are *Hermetia illucens* (HI) and *Tenebrio molitor* (TM), which, in addition to meeting the approval of the EU as feed ingredients, are produced in sufficient quantities to sustain animal feed manufacturing.

The effect of the inclusion of HI in aqua feed has been analyzed in relation to many fish species and many different issues, such as disease resistance, innate immune response, growth performance, intestinal antioxidant enzymes and amino acid composition [6]. Similar studies have been carried out for TM [7,8].

Sparus aurata, gilthead seabream, is a widely consumed fish in the Mediterranean area. The culture of *Sparus aurata* is expanding, as evidenced by 96% of its total production (aquaculture + fisheries) corresponding to aquaculture in 2016. In the period of 2012–2016, cultivated production increased by 32%, and an additional 14% from 2016 to 2017, reaching 94,936 tons and EUR 485 million [9]; this was mostly fueled by an increase in production in the Mediterranean Sea [9]. Currently, feeds for seabream are based in fishmeal and soy as protein sources. Nevertheless, sources of soy and fishmeal present environmental problems. To reduce their drawbacks, insect meal can be considered as a potential substitute due to its more sustainable nature and broad acceptance [10].

The inclusion of both insect species, HI and TM, in *S. aurata* feed has been studied previously [11]. Results pointed out the existence of a limit of substitution, 25–30%, above which growth indices worsened in comparison with fishmeal diets, although two recent studies by Pulido-Rodriguez et al. [12] and Randazzo et al. [13] reported similar or even better results with a level of substitution of 40% of vegetable protein mixed with HI meal. Nevertheless, higher percentages of substitution have been reported using other alternative protein sources, such as soy (40%) [14] or pea protein concentrates (60%) [15]. One of the main reasons for this limit of insect inclusion is attributed to the low digestibility of chitin and scleroprotein of insect exoskeletons [10]. Another handicap that limits the inclusion of insects in fish diets is the fatty acid profile, which is different from fishmeal. Insects have a higher percentage of n-6 polyunsaturated fatty acids (*n*-6 PUFAs), whereas fishmeal is richer in n-3 highly unsaturated fatty acids (*n*-3 HUFAs). In this sense, and trying to overcome this last challenge, Fabrikov et al. [16] obtained *n*-3 HUFAs-rich larvae feeding HI in discards from fisheries, and Liland et al. [17] produced similar results by feeding larvae with seaweed-enriched media.

Despite the performance of insect meal in relation to fish acceptance or its impact on growth parameters, the effects of high levels of inclusion on digestive and metabolic processes are not well known, and more knowledge is needed to realize the barriers precluding the use of insect meal in aquafeed. In this sense, it is also important to elucidate modifications in the gut microbiome due to the inclusion of insect meal in fish diets. Microbial communities associated with the intestinal tract play a decisive role not only in relation to nutrient assimilation, but regarding immunological responses and disease prevention [18]. Among these communities, some are considered as persistent and others are understood as transient [19], depending on their temporality. The presence of transient microbiota is subject to fluctuations caused by environmental factors, whereas persistent

communities are less affected by such conditions and only minor quantitative modifications can be observed. Among the most influential factors regarding the composition of transient microbiota are those of a nutritional nature [20]; these can promote the temporary presence of species more adapted to the properties of substrates used and, consequently, can foster the better utilization of a diet as a whole [21]. Thus, the study of the gut microbiome and its response to changes in the nutritional composition of feeds can help to interpret the specific roles of different species and even select potential probiotics adapted to the type of nutrients being used. Ultimately, this understanding might encourage the better use of alternative protein sources and allow for the partial and even full replacement of traditional fishmeal-based diets.

Considering the abovementioned discussion, the main aim of this study was to investigate the effect of high levels of replacement of fishmeal with TM or HI meal—including meal with a modified acid profile—on growth and nutritive indices, digestive and metabolic enzymes and the gut microbiota of *S. aurata* fed with such diets. Elucidating potential correlations between growth parameters and microbial communities was also established as a primary goal.

2. Materials and Methods

2.1. Insect Meals

HI and TM larvae used in this experiment were purchased from Entomotech S.L. and MealFood Europe S.L., respectively. One group of HI had been fed with fishery discards obtained from the port of Almería (Spain) to increase their nutritional value. Insects were dried and ground to obtain full-fat insect meal. Tables 1 and 2 show the proximal composition and aminoacidic profiles of the two HI and TM larvae, respectively. Methodologies applied for the determination of analyzed parameters are described in Section 2.4.1.

Table 1. Proximal composition of fresh insect meal.

g/100 g Fresh Matter	HI	Fish-Fed HI	TM
Fat (g/100 g)	25.6	27.8	27.0
Moisture (g/100 g)	8.0	8.7	5.0
Protein (g/100 g)	28.5	30.5	39.1
Ash (g/100 g)	9.75	8.66	3.42
Phosphorus (g/kg)	7.0	8.1	7.5
Calcium (g/kg)	35.2	27.0	0.93
Chitin (g/100 g)	7.5	7.7	5.9

HI: *Hermetia illucens*; TM: *Tenebrio molitor*.

Table 2. Aminoacidic profile of insect meal.

g/100 g Fresh Matter	HI	Fish-Fed HI	TM
Asp (aspartate)	2.92	2.84	3.71
Thr (threonine)	0.95	0.97	1.44
Ser (serine)	1.43	1.44	2.49
Glu (glutamate)	3.19	3.37	4.98
Pro (proline)	1.58	3.43	3.04
Gly (glycine)	1.84	2.02	2.87
Ala (alanine)	2.37	2.44	3.92
Val (valine)	1.42	1.46	2.32
Met (methionine)	0.47	0.41	0.57
Ile (isoleucine)	0.91	0.89	1.31
Leu (leucine)	1.86	1.83	2.96
Tyr (tyrosine)	2.23	2.05	4.47
Phe (phenylalanine)	2.16	1.81	3.07
His (histidine)	1.07	1.07	1.77
Lys (lysine)	1.94	1.87	2.49
Arg (arginine)	1.24	0.98	1.81

HI: *Hermetia illucens*; TM: *Tenebrio molitor*.

2.2. Diets

Five diets were prepared using different substitution levels of fishmeal by insect meal: the control diet (C) was prepared with fishmeal only, 30% substitution of fishmeal with HI meal (H30), 50% substitution of fishmeal with HI meal (H50), 50% substitution of fishmeal with fish-fed HI meal (H50M) and 50% substitution of fishmeal with TM meal (T50). For the H50M diet, HI larvae were fed with fish discards to modify their fatty acid profile by increasing their polyunsaturated fatty acid content. The formulated diets were isoenergetic, isoproteic and isolipidic. Table 3 shows the ingredients and proximal composition of the formulated diets.

Table 3. Ingredients and proximal composition of elaborated diets used in experiment.

Ingredients (g/100 g Fresh Matter)	C	H30	H50	H50M	T50
Fishmeal	35.9	25.3	18	18	18
HI meal	0	10.9	18	0	0
Fish-fed HI meal	0	0	0	18	0
TM meal	0	0	0	0	18
Wheat gluten	10.5	13	15.4	15	11.9
Soy protein concentrate	15.5	17.5	18.3	18.3	17
Wheat meal	16.4	13.4	11.5	11.7	17
Soy lecithin	1.3	1	0.5	0.5	0.5
Fish oil	12.2	10.4	9.5	9.7	9
Vitamins and minerals	2	2	2	2	2
Guar gum	2	2	2	2	2
Hemoglobin powder	4	4	4	4	4
Methionine	0.2	0.5	0.5	0.4	0.5
Lysine		0.1	0.4	0.4	0.1
Total	100	100	100	100	100
Proximal Composition (g/100 g Fresh Matter)					
Crude protein	43.9	43.5	42.8	42.9	43.1
Crude fat	17.2	17.6	17.1	17.6	18.0
Fiber	-	-	-	-	-
Ash	7.4	6.9	6.4	6.3	6.1

HI: *Hermetia illucens*; TM: *Tenebrio molitor*.

2.3. Fish Feeding Trial and Sampling

Juvenile *Sparus aurata*, with an average weight of 7.10 g (Wi, initial weight), were obtained from Predomar S.L. (Carboneras, Spain) and reared during the experimental period in the Aquarium of University of Almería (Almería, Spain). Fish were adapted to aquarium conditions over 15 days under constant temperature (20 ± 1 °C) and a natural photoperiod. After the acclimatation period, 450 fish were individually weighed and randomly distributed in 15 300 L experimental tanks (3 tanks/treatment; 30 fish/tank). For each treatment, three 300 L tanks were used. Fish were fed ad libitum twice a day at 09:00 and 14:00. Feed intake was recorded daily, and the experiment ended when fish fed on C diet tripled their body weight (32 days).

Before the end of the experiment, fish were fasted for 24 h prior to sampling. Fish were anaesthetized and weighed. For sample collection, 6 fish/tank were sacrificed with an overdose of anesthesia (clove oil, 100 mg/L); then, viscera were extracted and weighed. The liver, intestine, stomach and muscle were separated, weighed, frozen in liquid N_2 and stored at −80 °C until further analysis. To analyze apparent digestibility, feces samples were collected during the last two weeks of the growth trial by a modified Guelph method [22], gathering the feces produced throughout 24 h in a settling column. The feces samples were frozen at −80 °C until further analysis.

2.4. Sample Analysis

2.4.1. Proximal Composition

The nutritional profiles of insect meals, diets and fish used in all experiments were analyzed according to the standard procedures of the Association of Official Analytical Chemists [23], with specific methods as follows: crude protein content was determined by Kjeldahl (AOAC, 2005; #954.01) using a conversion factor of 6.25 for feeds, feces and muscle, and 4.67 for HI and 4.75 for TM; crude fat (EE) was determined by ethyl ether extraction (Soxhlet technique) (AOAC, 2005; #920.39); the moisture was gravimetrically quantified by drying at 105 ± 0.5 °C (AOAC, 2005; #934.01), whereas ash was gravimetrically determined after combustion at 500 °C in a muffle furnace (AOAC, 2005; #942.05) to a constant weight. Phosphorus quantification was achieved according to protocol ISO standard (1996) (ISO 13730) based on molecular absorption spectrophotometry (UV/Vis UV2, UNICAM, Cambridge, UK). Calcium was determined following the protocol described by Pessoa (2016) using the X-ray fluorescence method of dispersive energy (ED-XRF). Gamage and Shahidi's protocol (2007) was used for the obtention of chitin residue, which was washed with acetone, dried, and weighed. For the obtention of the amino acid profiles, hydrolysates from insect meal samples were obtained by mixing 1.5 mg with 200 µL of 6 N HCl for 22 h at 110 °C; these were then qualitatively and quantitatively analyzed by ion-exchange liquid chromatography and post-column continuous reactions with ninhydrin (Biochrom 30 AAA series, Biochrom, UK). The apparent digestibility of the protein was determined using acid-insoluble ash as a marker in feeds and feces [24].

2.4.2. Digestive Enzymes

Individual intestines and stomachs were homogenized in distilled water (1:1, w/v) with Polytron PT 2100 (Kinematica AG Inc., Lucerne, Switzerland) at 15,000 rpm in an ice bath. Extracts were centrifuged at 20,000× g (Orto Alresa Biocen 22r) at 4 °C for 20 min. Protein concentrations of extracts were measured with a Pierce™ BCA Protein Assay Kit (Thermo Scientific TM, Rockford, IL, USA) to obtain appropriate concentrations of enzymes for activity analysis. Acid protease activity was measured in stomach extract following the method described by Anson [25], and 0.5% hemoglobin was used as a substrate. Alkaline protease and alpha-amylase activities were measured from intestine extract. Alkaline protease activity was measured according to the method described by Walter [26], and alpha-amylase activity was measured following the method described by Somogyi-Nelson [27]; 1% casein and 1% starch were used as substrates, respectively. Absorbance was measured at 280 nm for protease and 560 nm for alpha-amylase using a Power Wavex microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) in duplicate in 96-well microplates (UV-Star Greiner Bio-One, Frickenhausen, Germany). Protease activity was described as the micrograms of tyrosine produced per minute and per milligram of protein. Alpha-amylase activity was described as the micrograms of maltose produced per minute and per milligram of protein at 37 °C.

2.4.3. Metabolic Enzymes

Individual livers were homogenized (1:9, w/v) in buffer solution (Tris/HCl 0.1 M, Triton X-100 0.1%, EDTA 0.25 mM and NaCl 0.01 M pH 7.6) with Polytron PT 2100 at 15,000 rpm in an ice bath. The mixture was centrifuged at 20,000× g for 30 min at 4 °C. Protein concentrations of extracts were measured with a Pierce™ BCA Protein Assay Kit (Thermo Scientific TM, Rockford, IL, USA) to obtain appropriate concentrations for each enzyme activity analysis.

Alanine aminotransferase (EC 2.6.1.2, ALT) was measured using the method described by Lain-Guelbenzu et al. [28] with L-alanine as a substrate. Aspartate aminotransferase (EC 2.6.1.1, GOT) was measured using the method described by Krista and Fonda [29] with L-aspartate as a substrate. Glutamate dehydrogenase (EC 1.4.1.2, GDH) was measured using the method described by Katoh et al. [30] with α-ketoglutarate as substrate. Pyruvate kinase (EC 2.7.1.40, PK), glucose 6-phosphate dehydrogenase (EC 1.1.1.49, G6PDH) and

fructose 1,6-bisphosphatase (EC 3.1.3.11, FPBase) were measured according to methods described by Furné et al. [31], using phosphonyl pyruvate, D-glucose 6-phosphate and D-fructose 1,6-biphosphate as substrates, respectively. Absorbance was measured at 340 nm using a Power Wavex microplate scanning spectrophotometer in duplicate in 96-well microplates. Activity of the enzyme was expressed as the amount of enzyme required to oxidize/reduce 1 µmol of NADH/NADP per minute and protein at 25 °C.

2.4.4. Gut Microbiota

Sequencing and bioinformatics were performed by Life Sequencing S.A. (Valencia, Spain). Gastrointestinal tract samples were processed in order to achieve the DNA extraction using the QiAamp power fecal Pro DNA kit (Qiagen Sciences, Germantown, MD, USA), according to the protocol described by Lyons et al. [32]. DNA extracts were tested for concentration and quality using a NanoDropTM 3300 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and used to construct the corresponding genomic libraries from the V3-V4 hypervariable region 16s rRNA gene [33], applying universal primers S-D-Bact-0341—b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′). Sequencing analysis was carried out on an Illumina MiSeq platform (Illumina, Ic., San Diego, CA, USA) utilizing a 300 bp fragments paired-end protocol. The amplification cycle was as follows: initial denaturation at 95 °C for 5 min, 25 cycles consisting of denaturation at 95 °C for 40 s, annealing at 55 °C for 2 min, extension at 72 °C for 1 min and a final extension step at 72 °C for 7 min. PEAR software v.0.9.1 (http://cme.h-its.org/exelixis/web/software/pear, accessed on 21 May 2021) was applied for the construction of the overlapped sequences and CUTADAPT software v.1.8.1 for the removal of adapters [34]. Sequences displaying a quality score higher than Q20, length > 200 bp and free-form chimeric sequences by the implementation of Chimera Uchime [35] were selected. The remaining quality sequences were clustered at a 97% cutoff using CD-HITt software v.4.6.8 [36,37] and phylogenetically classified to the maximum taxonomical level possible using the BLAST tool for Local Alignment (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 14 June 2021) and the associated GenBank database.

Shannon–Wiener and Simpson α biodiversity indices were calculated at species taxonomic level using a 97% sequence similarity threshold for the establishment of OTUs. Community similarity among samples (β biodiversity) was estimated by means of the Sørensen–Dice qualitative (presence/absence data) and quantitative (relative abundance data) indices.

2.5. Statistical Analysis

Three independent replicates were used in all analyses, and data are presented as the mean ± standard error. A one-way analysis of variance (ANOVA) and a multiple comparison of means based on the Tukey–Kramer HDS approach at a 95% confidence level were performed to test for significant differences between treatments regarding growth and enzymatic parameters. The relationships among all variables (growth, enzymatic and microbiological factors) and the existence of differences between samples were plotted through principal component analysis and correlation analysis. Correlations between variables were determined by Pearson product correlation at both 95% and 99% confidence levels. All statistical treatments were performed using IBM SPSS Statistics v. 26 software (IBM Analytics, Armonk, NY, USA) and Statgraphics Centurion XVI version 18.1.12 (Statpoint Technologies, Inc., Warrenton, VA, USA).

2.6. Ethical Standards

The experiment was conducted according to the directive 2010/63/EU of the European Parliament for the protection of animals used in experiments and for other scientific purposes and was approved by the Órgano Competente of Junta de Andalucía (n° ref.: 19/05/2017/065) according to Royal Decree 53/2013 of 1 February 2013.

3. Results

3.1. Growth and Nutritive Indices

Table 4 shows nutritional and growth indices worsened by the inclusion of insects, especially in the case of HI diets.

Table 4. Nutritional and growth indices and percentage of organs regarding final body weight of *Sparus aurata* fed with five experimental diets.

	C	H30	H50	H50M	T50
Wf	21.16 ± 0.70 ^{a}	14.00 ± 0.56 ^{c}	13.8 ± 0.15 ^{c}	14.90 ± 0.10 ^{c}	19.13 ± 0.15 ^{b}
DGC	2.48 ± 0.09 ^{a}	1.43 ± 0.09 ^{c}	1.40 ± 0.03 ^{c}	1.58 ± 0.02 ^{c}	2.21 ± 0.03 ^{b}
FI	3.10 ± 0.02 ^{a}	2.43 ± 0.01 ^{b}	2.48 ± 0.09 ^{b}	2.56 ± 0.12 ^{b}	3.16 ± 0.09 ^{a}
FCE	0.94 ± 0.02 ^{a}	0.79 ± 0.04 ^{bc}	0.76 ± 0.01 ^{c}	0.81 ± 0.03 ^{bc}	0.85 ± 0.01 ^{b}
PER	2.21 ± 0.03 ^{a}	1.85 ± 0.10 ^{bc}	1.79 ± 0.03 ^{c}	1.91 ± 0.04 ^{bc}	2.00 ± 0.03 ^{b}
%Viscera	9.95 ± 0.32 ^{a}	6.88 ± 0.44 ^{b}	6.71 ± 0.58 ^{b}	7.83 ± 0.39 ^{b}	10.29 ± 1.02 ^{a}
%Stomach	1.31 ± 0.09 ^{a}	0.94 ± 0.01 ^{b}	0.98 ± 0.15 ^{b}	0.99 ± 0.10 ^{b}	1.16 ± 0.15 ^{ab}
%Intestine	3.93 ± 0.51 ^{a}	2.95 ± 0.03 ^{bc}	2.81 ± 0.37 ^{c}	3.28 ± 0.27 ^{abc}	3.74 ± 0.01 ^{ab}
%Liver	1.92 ± 0.19 ^{a}	1.30 ± 0.11 ^{b}	1.15 ± 0.10 ^{b}	1.43 ± 0.12 ^{b}	1.96 ± 0.21 ^{a}
%Visceral fat	1.83 ± 0.24 ^{a}	1.43 ± 0.48 ^{b}	1.12 ± 0.14 ^{b}	1.19 ± 0.06 ^{b}	2.09 ± 0.30 ^{a}

Means (±SE) of three replicate tanks (30 fish/tank). Wf, final body weight; Wi, initial body weight (average value = 7.10 g); DGC (daily growth coefficient) = [(Wf1/3–Wi1/3)/t] × 100); FI (feed intake) = (daily feed intake/average body weight) × 100; FCE (feed conversion efficiency) = wet weight gain/dry feed intake; PER (protein efficiency ratio) = wet weight gain/crude protein intake. Different letters indicate significant differences ($p < 0.05$) based on Tukey–Kramer HSD test.

In statistical terms, treatments could be categorized into three groups: control treatment, which showed the best result; the three experimental diets with HI at any percentage of replacement or the use of modified meal; and the T50 diet. Nevertheless, nutritional indices, such as FCE and PER, did not show significant differences among H30, H50M and T50 treatments. On the other hand, and with respect to morphological indices (Table 4), the use of HI meal as an alternative protein source produced significantly lower responses, except for intestine percentage, whereas differences were not found between C and T50 diets. In this case, the results were not always statistically different, because values for H50M were similar to that of C.

Regarding proximal composition of the muscle (Table 5), a similar protein content was found between treatments. A similar pattern was observed in the case of lipid content, although differences were high enough to generate significant differences. Moisture also varied between treatments; lower moisture values were found for the control diet and the highest moisture was observed in the H50M diet. Nevertheless, statistical differences only appeared between control and H30 and H50 diets. Ash contents increased with HI content in the diets, showing statistical differences between the diet using HI and the other diets, except for C and H30.

Table 5. Proximal composition of raw muscle and apparent digestibility coefficient of diet protein from *Sparus aurata* fed with experimental diets. All results are expressed as percentages.

	Moisture	Fat	Protein	Ash	$ADC_{protein}$
C	71.93 ± 0.67 ^{a}	4.70 ± 0.55 ^{ab}	19.63 ± 0.43 ^{a}	1.88 ± 0.06 ^{ab}	93.86 ± 0.26 ^{b}
H30	73.93 ± 0.29 ^{b}	3.66 ± 0.52 ^{ab}	18.70 ± 0.61 ^{a}	2.14 ± 0.06 ^{b}	79.13 ± 1.64 ^{a}
H50	73.66 ± 0.27 ^{ab}	3.06 ± 0.12 ^{a}	18.83 ± 0.22 ^{a}	2.76 ± 0.04 ^{c}	79.16 ± 0.25 ^{a}
H50M	74.26 ± 0.12 ^{b}	3.60 ± 0.35 ^{ab}	19.16 ± 0.39 ^{a}	2.57 ± 0.06 ^{c}	91.57 ± 0.28 ^{b}
T50	72.43 ± 0.51 ^{ab}	5.56 ± 0.54 ^{b}	18.30 ± 0.21 ^{a}	1.77 ± 0.08 ^{a}	93.20 ± 0.23 ^{b}

Means (±SE) of three replicate tanks (30 fish/tank). $ADC_{protein}$ (apparent digestibility coefficient of the protein). Different letters indicate significant differences ($p < 0.05$) based on Tukey-Kramer HSD tests.

The apparent digestibility coefficient (ADC) showed lower values for HI-based diets than for C or TM, except for H50M.

3.2. Intermediary Metabolism

No significant differences in terms of enzyme activity related to glucose metabolism (Figure 1A) were detected for any of the enzymes determined (PK, pyruvate kinase; FBPase, fructose-1,6-biphosphatase; G6PDH, glucose-6-phosphate dehydrogenase) between different treatments. Regarding enzymes of amino acid metabolism (Figure 1B), results associated with GDH (glutamate dehydrogenase) activity did not give rise to different statistical groups either, whereas those corresponding to GPT (glutamate pyruvate transaminase) and GOT (glutamic oxalacetic transaminase) were divided into two significant groups constituted by, on one hand, HI-based diets, and on the other hand, T50. C treatment remained in an intermediate position, associated with both groups.

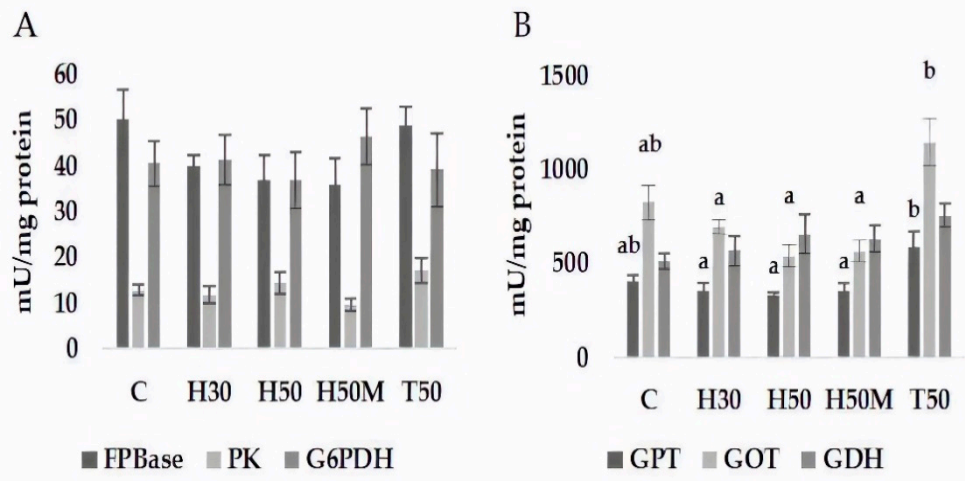

Figure 1. Enzymes of intermediary metabolism of *Sparus aurata* fed with experimental diets. Means (±SE) of three replicate tanks (30 fish/tank). Different letters indicate significant differences ($p < 0.05$) based on Tukey–Kramer HSD tests. Columns without letters indicate an absence of significant differences. Sugar-related enzymes are depicted in Subfigure (**A**) (FPBase (fructose-1,6-biphosphatase); PK (pyruvate kinase); G6PDH (glucose-6-phosphate dehydrogenase)) and amino acid-related enzymes in Subfigure (**B**) (GPT (glutamate pyruvate transaminase); GOT (glutamic oxalacetic transaminase); GDH (glutamate dehydrogenase)).

3.3. Digestive Enzymes

Alpha-amylase and alkaline protease activities (Figure 2) showed similar behavioral patterns, with maximal and minimal levels for H50 and H30 treatments, respectively, and higher values for H50M and T50 in comparison to the C diet.

Regarding acid protease (Figure 2), the inclusion of insect meal had a clear negative effect on the activity of this enzyme, with reductions of around 50%. In contrast, no significant differences were observed between diets including insect meal.

3.4. Effect of Insect Meal Inclusion on Bacterial Communities

The inclusion of insect meal in fish diets caused changes in the microbial communities associated with the gastrointestinal tract. Regarding the number of operational taxonomic units (OTUs), such changes showed different trends depending on the insect and the percentage of its inclusion (Table 6). Thus, whereas H30 and T50 promoted an increase in the total number of OTUs, the use of H50 and H50M gave rise to a decrease; this was especially noticeable in the last case. Curiously, a higher number of OTUs did not always correspond to greater diversity expressed by means of the Shannon–Wiener index, because H50M showed the second highest diversity, whereas the minimal value was detected for T50. The values obtained for the Simpson index confirmed this scenario, allocating the lowest level, i.e., greater homogeneity, to T50 samples. It is worth mentioning the case of H30. Curiously, fish fed with this diet showed a more diverse and heterogeneous microbiome than C, but only when the whole community was estimated. In contrast, when

only prevalent OTUs were considered (relative abundance > 0.5%) both diversity and heterogeneity were much lower than in the C diet.

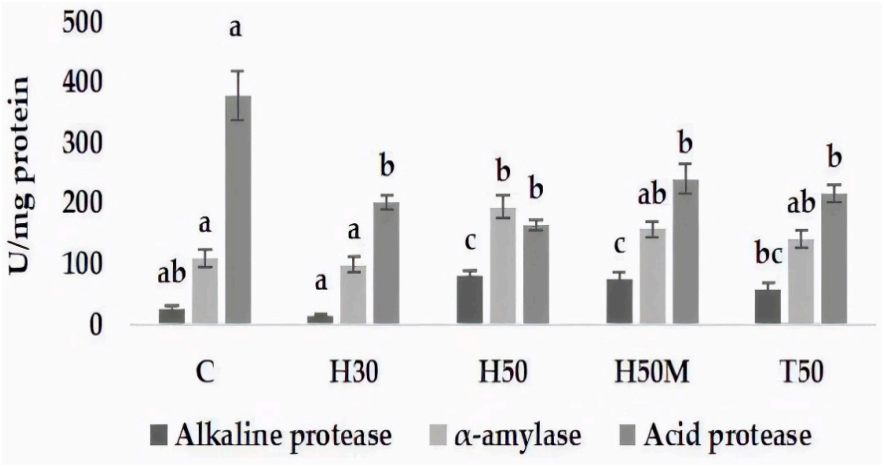

Figure 2. Digestive enzymes of *Sparus aurata* fed with experimental diets. Means (±SE) of three replicate tanks (30 fish/tank). Different letters indicate significant differences ($p < 0.05$) based on Tukey–Kramer HSD tests.

Table 6. Number of OTUs and values for some α biodiversity indices observed for different treatments. Biodiversity indices are shown for the entire community, as well as for the community comprising those OTUs representing 0.5% of the relative abundance in at least one of the samples.

	C	H30	H50	H50M	T50
Total number of OTUs	281 ± 67	335 ± 60	270 ± 23	206 ± 22	308 ± 45
Shannon-Wiener richness index					
Entire population	2.59 ± 0.94	3.10 ± 1.55	2.39 ± 0.98	2.85 ± 1.13	2.16 ± 1.44
OTUs > 0.5%	2.15 ± 0.77	1.53 ± 0.92	2.53 ± 0.77	2.34 ± 0.63	2.06 ± 0.99
Simpson dominance index					
Entire population	0.66 ± 0.18	0.79 ± 0.30	0.70 ± 0.15	0.77 ± 0.31	0.49 ± 0.27
OTUs > 0.5%	0.63 ± 0.18	0.42 ± 0.25	0.76 ± 0.28	0.74 ± 0.14	0.68 ± 0.26

Regarding similarities between treatments, expressed by the β Sørensen-Dice diversity index, correspondences between C samples and the rest remained in the 45–50% range (Table 7). From a quantitative perspective, considering the relative abundance of each OTU, the diet that promoted maximal alteration in the composition of the prokaryotic community was H30, whereas H50M preserved the greater percentage of similarity. When comparisons were limited to those OTUs presenting a relative abundance over 0.5% in at least one of the treatments (restricted community), qualitative results increased to levels over 80% in most cases, although quantitative values remained virtually unchanged. Thus, H50M and H30 again showed maximal and minimal quantitative similarities with the C treatment (65% and 27%), values which were nearly identical to those found when the whole population was considered. The highest and lowest qualitative similarity were observed for H30/H50M (97%) and H50/H50M pairs (78%), respectively, whereas such role corresponded to H50/T50 (80%) and H30/C pairs (27%) when quantitative data were analyzed.

Table 7. β Diversity indices (Sørensen-Dice) observed for the entire (A) and restricted communities (B). Qualitative results are depicted in the upper quadrant and quantitative in the lower quadrant.

(A)	C	H30	H50	H50M	T50	(B)	C	H30	H50	H50M	T50
C		0.45 ± 0.05	0.45 ± 0.06	0.48 ± 0.05	0.49 ± 0.09	C		0.89 ± 0.09	0.83 ± 0.07	0.89 ± 0.10	0.89 ± 0.09
H30	0.27 ± 0.12		0.42 ± 0.07	0.49 ± 0.02	0.60 ± 0.05	H30	0.27 ± 0.11		0.80 ± 0.42	0.97 ± 0.36	0.92 ± 0.33
H50	0.50 ± 0.25	0.56 ± 0.26		0.42 ± 0.05	0.49 ± 0.07	H50	0.52 ± 0.30	0.58 ± 0.33		0.78 ± 0.08	0.83 ± 0.07
H50M	0.64 ± 0.25	0.39 ± 0.28	0.59 ± 0.25		0.51 ± 0.04	H50M	0.65 ± 0.14	0.40 ± 0.27	0.61 ± 0.26		0.89 ± 0.10
T50	0.56 ± 0.25	0.67 ± 0.30	0.79 ± 0.23	0.60 ± 0.21		T50	0.56 ± 0.17	0.69 ± 0.34	0.80 ± 0.23	0.61 ± 0.21	

A more in-depth analysis of this predominant community (relative abundance over 0.5%) (Figure 3) revealed the existence of two main clusters, one composed of C and H50M treatments and the other comprising H30, H50 and T50 samples, with the last two forming a subcluster. All the OTUs found were assigned to Firmicutes and Proteobacteria phyla, with *Bacillus* and *Vibrio* representatives prevailing in all cases. Members of the Bacillales order and the *Vibrio harveyi* group contributed a higher percentage to the C/H50M microbiome cluster, whereas *Vibrio tubiashii* and *Vibrio* sp dominated the prokaryotic community of the H30/H50/T50 cluster. From a global perspective, the inclusion of insect meal, whatever its form, produced a decrease in the relative abundance of *Weissella confusa*, *Streptococcus dysgalactiae* and *Peptostreptococcus russelliii*, as well as the emergence of *Sulfitobacter pontiacus* and representatives of the Alteromonadaceae family.

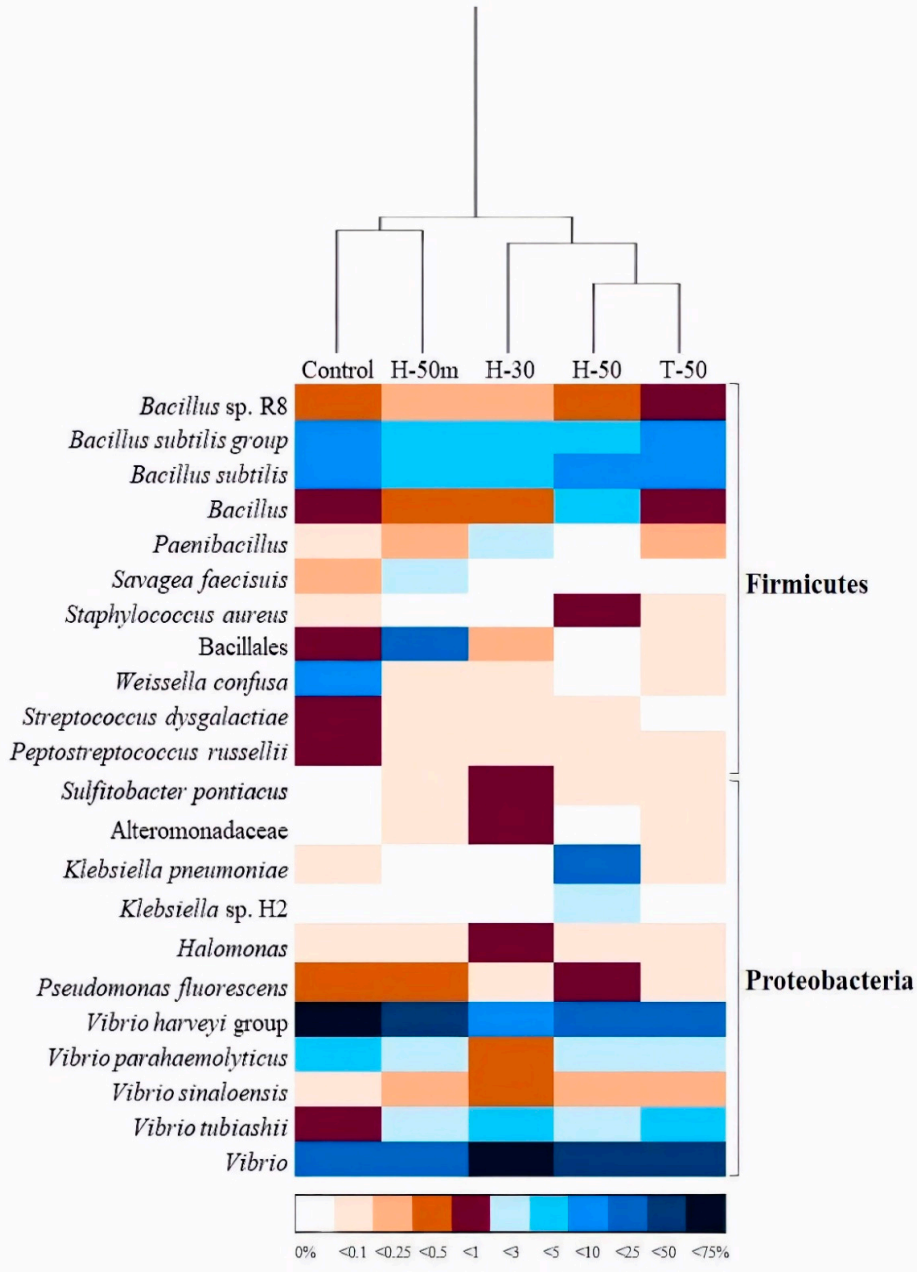

Figure 3. Heatmap representing the distribution, expressed as a percentage, of OTUs within samples and a dendrogram showing the relationships between treatments based on a restricted microbial community.

3.5. Interrelationships between Microbial Community and Fish Parameters

Figure 4 shows results obtained from the principal component and discriminant function analyses. The treatments were divided into two main groups: the first one made up of C and T50 diets, and the second one formed of treatments including HI meal, with a subgroup integrated by H50 and H50M. The first group was related to all biometric parameters and most of the metabolic enzymes, whereas the associated bacterial community was mainly dominated by Gram-positive species. On the contrary, the parameters connected to the second group were limited to two of the digestive enzymes (alpha-amylase and alkaline protease) and one metabolic enzyme (G6PDH), as well as members of the Proteobacteria phylum with respect to the assignation of the prevalent species. The latter was particularly noticeable in the case of the H50 treatment.

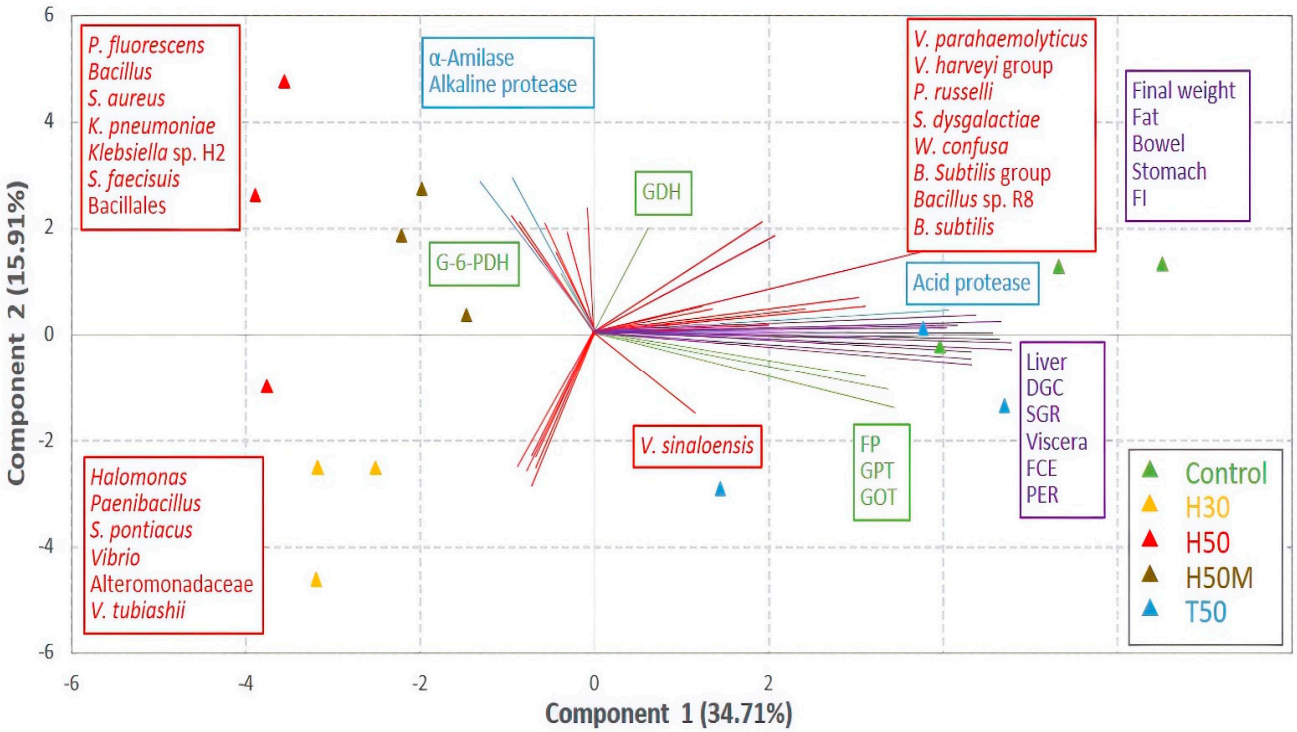

Figure 4. Principal component analysis biplot showing grouping of treatments and relationships amongst variables as influenced by treatments. Metabolic enzymes are depicted in green, digestive enzymes in blue, biometric parameters in violet, and microorganisms in red.

Specific connections between parameters were better reflected in the correlation analysis (Figure 5). According to the results, all biometric parameters were closely interrelated. Enzymes such as alpha-amylase, acid protease, FBPase, GPT and GDH also showed strong correlations with most of the biometric parameters. Regarding the microbial community, two species were relevant on account of their influence on growth and nutritive indices: *Streptococcus dysgalactiae* and *Peptostreptococcus russellii*. *Weisella confusa*, in contrast to these two bacteria, was positively correlated with two significant indices, as was the case with FCE and PER. However, *Sulfitobacter pontiacus*, *Halomonas* and Altermononadaceae showed a strong negative correlation with the final weight.

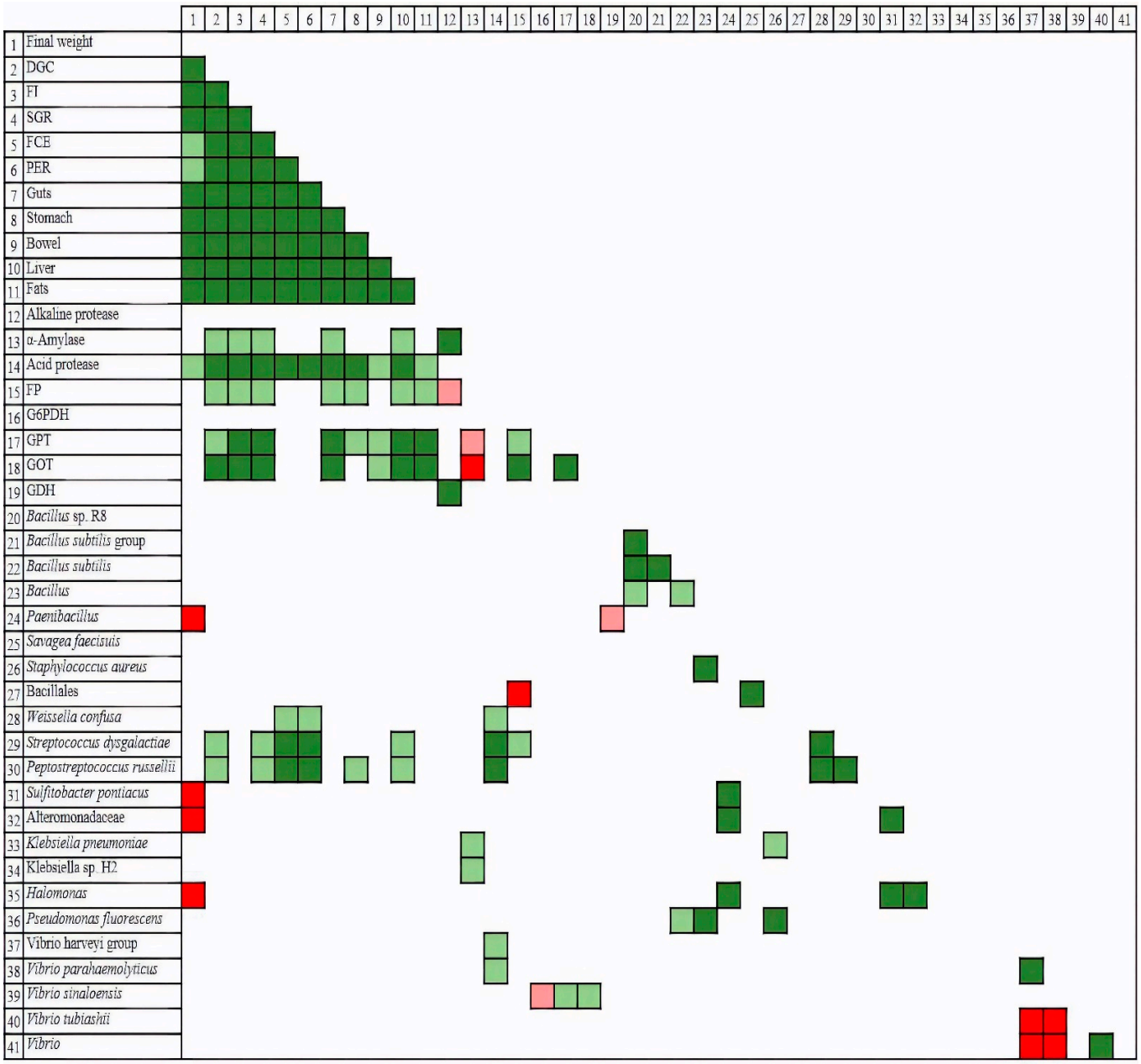

Figure 5. Correlations between parameters according to Pearson correlation analysis: positive results are depicted in green and negative results in red. Dark and light colors reveal significance at 99% and 95% confidence levels, respectively.

4. Discussion

In recent years, the replacement of fishmeal in aquafeed has been studied widely. Specifically, in feed for *S. aurata*, the level of inclusion of HI or TM meal without compromising growth has been established as being over 25–30% [11,38]. Higher replacement percentages need to be studied thoroughly to deepen knowledge regarding the digestive and metabolic responses of fish to insect meal ingestion. In this experiment, diets with high levels of substitution of fishmeal with insect meal (50:50) were studied, as was HI meal with a modified fatty acid profile according to the method described by Fabrikov et al. [16].

Nutritional and growth indices (Table 4) showed better results for the control diet in all the indices determined, followed by T50. Piccolo et al. [38] obtained similar growth rates for *S. aurata* fed with a fishmeal-based diet and a diet including a 25% replacement with TM meal, which was lower than that in our experiment. Pulido-Rodriguez et al. [12] and Randazzo et al. [13] also obtained similar results with inclusion of levels of 32.4%. Other

than different percentages of fishmeal substitution, these discrepancies might be related to varying specific growth rates, which were around 2 in our experiment versus values closer to 0.5 in Piccolo et al. [35] and to 1.5 in Pulido-Rodriguez et al. [12] and Randazzo et al. [13]; these values are in accordance with the different initial weights used, i.e., 100 g and 48.8 g, respectively, versus 7 g in our experiment. FCE and PER also worsened in the T50 diet in comparison to C treatment, whereas no differences were observed in the study by Piccolo et al. [38] at a replacement level of 25%.

Fish fed with HI meal, no matter the level of replacement, showed worse nutritional and growth indices, which agrees with results obtained by Karapanagiotidis et al. [11]. Nevertheless, values observed for FCR and PER in the cases of H30 and H50M diets seem to be slightly better than those from H50. In the case of H50M, this result might be a consequence of the improved fatty acids profile of HI; however, in the case of H30, it could be due to the lower level of insect inclusion. The influence of insect meal on growth can also be illustrated with morphometric indices (Table 4). Results obtained for these indices confirmed what the nutritional parameters pointed out, with HI diets producing lower values. However, no differences between C and T50 treatments were found in this case, which support the idea of the better nutritive quality of TM meal in comparison with HI for *S. aurata* juveniles, especially when the latter does not improve nutritional profile.

Data regarding HI substitution levels higher than 30% in *S. aurata* feeding are scarce, if non-existent, although some studies have been published with other species as subjects. Thus, Reyes et al. [39] described similar results for *Dicentrarchus labrax*, reporting a better acceptance of diets containing a 30% inclusion in comparison to those with a 50% replacement. In contrast, no differences in nutritional and growth indices were detected for growth responses between fish fed with diets including 30% HI meal or 50% TM meal, showing a slightly better nutritive utilization of HI by *D. labrax* than by *S. aurata*.

The response of fish to specific diets is dependent on digestibility, which, in turn, is primarily associated with protein assimilation [38,40]. As such, the differences in growth and nutritional indices between HI- and TM-based diets could be due to the different digestibility of feeds obtained for *S. aurata* (CDA H30: 79.13; CDA H50: 79.16; CDA T50: 93.20). These values of digestibility are in agreement with those obtained in *D. labrax* for HI [6] and higher than those obtained in TM [41]. The different digestibility in these two insect species contrast with those reported by Marono et al. [42], who obtained an in vitro crude protein digestibility lower than in the present study but that was similar among different samples of HI and TM. Chitin content could also affect digestibility, and TM meal contains less chitin than HI meal (Table 1), which may partly explain the different digestibility indices measured in each case. The high CDA found for H50M (CDA H50M: 91.57) might be attributable to microbiological factors, because chitin concentrations must be similar. According to the results, H50M-fed fish showed the most similar microbiome to those undergoing C treatment, and both were different from the rest of the diets. Such differentiation was mostly based on the higher population of members of the genus *Bacillus*, especially, to those belonging to the *Bacillus subtilis* group, whose chitinolytic properties are well recognized [43].

On the other hand, digestibility is also related to digestive enzyme activities. In this respect, the results indicate that insect meals induced changes in digestive enzyme activities, decreasing the activity of acid protease and increasing alkaline protease and alpha-amylase, especially for H50 and H50M. In trout fed with HI or TM meal at 15% or 30% fishmeal replacement, an increase in alkaline protease activity was also observed, although no affectation of acid protease activity was detected [44]. Conversely, decreases in alkaline protease and trypsin activities were described in *Argyrosomus regius* fed with a diet with 30% fishmeal replacement by TM [45], whereas the inclusion of HI in *D. labrax* feed did not affect intestinal protease activities [6]. The digestibility of protein from insects can be highly variable, depending on the proportion of chitin [40]. Coutinho et al. [45] explain these changes as a slowdown in intestinal transit due to chitin that causes decreases in digestive enzyme activities. Nevertheless, protein digestibility is mostly influenced

by the amino acid contents in ADF fractions [46]. Hence, changes in digestive protease activities could be associated with the skleroprotein of an insect's exoskeleton, in addition to the chitin effect, as proposed by Coutinho et al. [45]. On the other hand, there is a clear connection between enzyme activity and dietary habits [47]; both may influence and be influenced by the intestinal microbiome [48]. Thus, the detection of different enzyme responses due to changes in feed and microbial communities should not be surprising.

Alpha-amylase activity showed a tendency to increase activity at high levels of insect inclusion. The results described by other authors vary among fish or insect species. For *D. labrax* fed with a 45% replacement with HI [6], or *Argyrosomus regius* fed with a diet with 30% fishmeal replacement with TM [45], no changes in alpha-amylase activity were detected; however, in *Oreochromis mossambicus* and *Clarias gariepinus* fed with *Imbrasia belina*, an increased amylase activity was found [49]. In rainbow trout *(Oncorhychus mykiss*), the inclusion of 30% HI meal produced a reduction in alpha-amylase activity, whereas no effect was observed when TM meal was used. Reasons for such varying responses are not clear, especially due to the absence of data dealing with 1,4-α-glucose polymer content, i.e., the substrate for this enzyme.

On the other hand, the inclusion of insect meal as an ingredient in diets did not seem to alter the intermediary metabolism of glucose or amino acids, because none of the analyzed enzymes involved in such processes showed significant changes in comparison to the C diet. Similar results were found for rainbow trout [44] and sea trout (*Salmo trutta* m. *trutta*) at low levels of replacement of fishmeal with TM meal [50]. Insect species induced changes in the Vmax of GPT between HI treatments regarding T50. Fabrikov et al. [5] studied the adaptation of amino acid catabolism enzymes to HI or TM meal inclusion in the diet among different species, including *S. aurata*. They found that Vmax exhibited an increase no matter the insect used for the formulation of the diet. However, in the present study, results showed different trends related to different insect species. Thus, although TM replicated the results from Fabrikov et al. [5], i.e., the Vmax of GPT increased in value, HI tended to reduce activity. In the case of GOT, Fabrikov et al. [5] did not find changes in the Vmax when HI meal was included in the diet, whereas an increase was observed when fishmeal was partially substituted by TM meal. These different responses could be attributed to the inclusion level—15% and 30% replacements in the experiment by Fabrikov et al. [5] and 50% in this assay—which might have led to a reduction in feed intake in HI groups. This fact, as well as lower digestibility, potentially reduces the availability of amino acids to be deaminated and, consequently, decreases GOT activity. In T50-fed fish, feed intake is higher than in HI groups, with the subsequent increase in amino acid availability being utilized as energy or for gluconeogenesis purposes, promoting both transaminase activities (GPT and GOT).

4.1. Gut Microbiome Response to Dietary Insect Inclusion

Previous studies have reported variations in the gastrointestinal microbiota of fish due to modifications in their diets [51]. In this case, the shaping of the microbiota is a logical response of the gut environment to variations in the nutritional composition of the diet, given the plasticity of bacterial communities and their rapid adaptation to new conditions [21]. In relation to the partial replacement of fishmeal with insect meal, some studies described an increase in the diversity of the intestinal microbiome [52], although opposite results have been obtained depending on insect and fish species, insect life-cycle stage or replacement level [53,54]. In the present work, different responses seem to be preferably related to the level of replacement rather than insect species, as revealed by the β diversity. Curiously, greater differences with regard to C diet were detected at the lowest level of inclusion, H30, which may seem contradictory, although this result has previously been reported [53]. Microbial community structures are dependent on several factors, and the characteristics of diet is just one of them. Environmental, phylogenetic and physiological interactions come in a wide range of conditions that, in turn, can create particular habitats which affect and modulate the composition of the gut microbiome [55].

In this sense, insect meal shows properties that can affect its acceptance by fish [56], as is the case of chitin content and fat profile. Regarding the latter, HI is characterized by a high saturated fatty acid content, as well as an unbalanced fatty acid profile [54] that can negatively affect fish performance. Therefore, it is common practice to provide defatted or fatty-modified meals with the potential to reduce undesirable effects and improve meal composition [16]. Concerning the gut microbiome, our results seem to suggest that such treatments may promote a nutritional profile more akin to that of fishmeal, which caused a lower impact in bacterial communities in the intestinal tract. Therefore, these two diets, C and H50M, clustered together. The almost complete lack of studies comparing the effect of full-fat and defatted insect meal on the fish gut microbiome has made it difficult to validate such a hypothesis, although it has been described in relation to other species [57]. Moreover, several documents point out the higher nutritional quality and digestibility of meals with a modified fat profile [58].

The dominance of *Vibrio* and *Bacillus* representatives in the fish gut microbiome is a common reality [19]. Members of the *V. harveyi* group, which is dominant in the C/H50M cluster, are associated with pathogenic processes [59]. Thus, the handover from this group to *Vibrio* sp. in the H30/H50/T50 cluster can be considered as positive, because species other than pathogens have been described in the genus, as in the case of *V. alginolyticus* [60], which has been postulated as a probiotic in aquaculture. The second largest microbial group was the *Bacillus* genus and its relatives. The importance of these bacteria relies on their enzyme activity and, to a lesser extent, their immunostimulant ability and antimicrobial production [61]. Taking into account their beneficial activities, and their presence in all treatments, it is clear that they have a positive impact on fish performance and wellbeing.

Apart from these dominant groups, other species were differentially present in the intestinal tract because of insect meal replacement. Bacteria such as *Streptococcus dysgalactiae*, *Peptostreptococcus russellii*, and especially *Weisella confusa*, reached relatively important population densities in the C samples, although they were practically absent in the rest of the treatments. *W. confusa* is a lactic acid bacterium which is associated with some infectious processes in human beings [62], but not in fish [63]. Moreover, it has been postulated as a probiotic for some species in aquaculture [64]. *P. russellii* has been positively associated with intestinal homeostasis in different species [65], although there is no literature about this in relation to fish. In contrast, *S. dysgalactiae* have been described as pathogenic for both human and fish [66]. However, *Sulfitobacter pontiacus* and members of the family Alteromonadaceae, absent in C-fed fishes, were detected in insect meal samples, especially H30. The first is a recognized sulfite and thiosulfate-oxidizing bacterium that might promote organic matter degradation in aquatic environments [67], whereas the latter comprises both pathogenic and potentially probiotic species [68,69].

In short, the replacement of fishmeal with insect meal seems to promote a redistribution of the most dominant bacterial populations, Vibrionaceae and Bacillaceae, whereas it modifies the identity of minor species. Nevertheless, and taking into account the diversity of conditions in which assays were performed, more information dealing with the impact of dietary modifications on intestinal bacterial communities is needed to attain a better understanding of the particular factors that rule the microbial response.

4.2. Gut Microbiome/Fish Performance Correlations

Gut microorganisms play different roles with respect to host performance and health. Beneficial bacteria can excrete enzymes that contribute to assimilating nutrients, reducing the availability of attachment sites for pathogens and synthesizing antimicrobial compounds [18]. The so-called persistent microbiota, which comprise those bacteria residing permanently in the gut, are considered as main protagonists of the symbiotic relation between the gut microbiome and a host [70]. Transient species, mostly associated with external environmental and nutritional factors, are thought to play some significant roles depending on their colonization ability [71]. Thus, species able to survive the conditions in the gut and adhere to intestinal mucosa may become permanent residents [72] and perform

primary functionality [73]. The result described in this paper showing the grouping of T50 and C samples, and leaving treatment most microbiologically similar to C, H50M, out of this group, may be explained by this theory. Moreover, the evolutionary closeness between species in T50 and C, which shows the possibility of sharing metabolic properties, sustains such a hypothesis. The positive relationship between both diets and fish performance was evident by means of pooling with all the biometric parameters and most of the metabolic enzymes. Relevant microorganisms in the gut microbiome of these treatments, such as the *Bacillus subtilis* group, were also in this group, as well as *W. confusa*, *S. dysgalactiae* and *P. russellii*. These latter three were statistically correlated with many of the biometric parameters, such DGC, SGR, FCE and PER, reflecting their involvement in the beneficial utilization of a diet by fish. Thus far, none of them have been considered as key players in fish performance. *S. dysgalactiae* has even been associated with pathogenic processes [66]. Nevertheless, it has been reported that this bacterium could be a member of the persistent gut microbiome in fish, although its specific origin is not yet clear [74]. On the other hand, it has been referenced as a bacteriocin-producing bacteria [75], which is a desirable characteristic on account of its ability to prevent the growth of some other pathogens. For its part, *W. confusa*, which is also an antibacterial compound producer [76], has shown potential to positively impact fish growth performance, as well as stimulate the activity of some digestive enzymes [77]. Similarly, *P. russelli* has been associated with beneficial effects in terms of fish health and growth, especially regarding activities dealing with protein degradation [78], as elucidated in this paper.

5. Conclusions

The partial replacement of fishmeal with insect meal has proved to be an effective strategy, although the results depend on insect species and their characteristics, as well as the percentage of replacement. The impact of meal replacement was also different according to the nature of the parameters considered. Thus, growth, nutrition and enzymatic parameters seem to be more affected by the insect species used for producing the meal, whereas the composition of the microbial community associated with the gastrointestinal tract is likely to be mostly influenced by the level of replacement and the pretreatment of insects. Thus, *T. molitor* meal (T50) caused the lesser degree of impact on growth performance as indicated by biometric and enzyme parameters. On the other hand, modified *H. illucens* meal (H50M) was the only treatment in which, for the most part, the microbial community structure was maintained. In addition, the results seem to assign a relevant role to certain minority species (*Peptostreptococcus russellii*, *Streptococcus dysgalactiae* and *Weisella confusa*) not related to fish gut microbiota thus far. These results are not conclusive, because different species are approved for use in aquaculture feed and conditions in which insects are reared and meals are obtained differ between trials; however, this study contributes to the subject and establishes the most adequate conditions to promote insect meal-based feed utilization in fish.

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CAPÍTULO V: *Antimicrobial and antioxidant activity of encapsulated tea polyphenols in chitosan/alginate-coated zein nanoparticles: A possible supplement against fish pathogens in aquaculture*

Antimicrobial and antioxidant activity of encapsulated tea polyphenols in chitosan/alginate-coated zein nanoparticles: A possible supplement against fish pathogens in aquaculture

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Abstract

Due to the increase of aquaculture facilities, where a large number of animals live in a relatively small area, infectious diseases expanded, resulting in large losses in the sector. These infections not only affect the farmed fish but also spread the pathogens to the ecosystem. Regulation of the use of antibiotics calls for the emergence of more sustainable alternative treatments. Epigallocatechin gallate (EGCG) is a secondary metabolite present mainly in the leaves of *Camellia sinensis* with diverse biological activities. However, EGCG is very susceptible to degradation, reducing its absorption in the digestive process.

In this work, EGCG and green tea extract were encapsulated in zein nanoparticles stabilized with alginate and chitosan to reduce the degradation effect. For all formulations, nanoparticles with a hydrodynamic size of less than 300 nm and an absolute ζ -potential value >30 mV were obtained. The encapsulation efficiency gave values above 75% for the polysaccharide-stabilized particles. The antioxidant capacity (DPPH and ABTS assays) of the encapsulated substances, although lower than the free ones, maintained high levels, SC50 of 33.6 and 63.3 μg/mL for encapsulated EGCG and GTE respectively was found. On the other hand, the evaluation of the antimicrobial activity, tested against five fish pathogenic bacteria, showed greater efficiency in terms of growth inhibition for nanoparticles with chitosan, with average overall values of around 60 %, although in the specific case of *Photobacterium damselae*, the most sensitive species, inhibition levels of over 90 % were recorded. These results support encapsulation as a good strategy for compounds of polyphenolic nature, since it allows maintaining significant levels of antioxidant activity and increasing the potential for antimicrobial activity, in addition to conferring protection against the hostile conditions they may face in their application in the aquaculture sector.

Keywords: aquaculture, green tea polyphenols, antibacterial, antioxidant, nanoparticles

1. Introduction

Aquaculture reached in 2020 46 % of the total fish production industry. Since the 1990s, aquaculture has grown 300 %, reaching a production of 87.5 million tons (FAO, 2022). However, this fast growth of the aquaculture industry brought several challenges, such as the elimination of pathogens (Pérez-Sanchez et al., 2018), feed utilization (Encarnação, 2016), contamination (Ton Nu Hai et al., 2020) and sustainability (Boyd et al., 2020).

Infectious diseases in aquatic animals cause a loss of 6 billion US dollars per year (Stentiford et al., 2017); this amounts to 50 % of the aquaculture industry located in developing countries (Assefa and Abunna, 2018). Diseases affect the growth performance, increasing the mortality and lowering the marketability of affected animals, which is exacerbated by saturated and stressful environments such as aquaculture productions (Lafferty et al., 2015). Bacterial infections in aquatic animals are mainly caused by Gram-negative bacteria as *Aeromonas* spp, *Vibrio* spp, *Pseudomonas* spp, etc., and Gram-positive bacteria *Streptococcus* spp and *Staphylococcus* spp., etc (Preena et al., 2020). Infectious diseases not only affect aquaculture production but increase the spread of pathogens to wild animals which causes an impact in capture industry and aquatic ecosystem (Diana, 2009).

Antibiotics have been one of the main chemicals used in aquaculture for combating infectious diseases, although in recent years their use have been diminished due to the increase of antibiotic resistant bacteria and the regulation of their use (Regulation (EU) 2019/6), thus limiting the number of antibiotics allowed and cases where it can be applied (Lulijwa et al., 2019). New strategies for combating or preventing infections have been emerging in recent years such as vaccines (Mondal and Thomas, 2022), feed supplemented immunostimulants (Wang et al., 2017a), nanomaterials (Okeke et al., 2022), etc.

Polyphenols are secondary metabolites produced as a stress response by plants. Over 10,000 polyphenols can be found in nature and can be classified by their structure as flavonoids (catechins, quercetin, curcumin) and non-flavonoids (gallic acid, cinnamic acid, resveratrol). Polyphenols have a wide range of biological activities, and several studies showed their antibacterial, antioxidant, growth promoter, anti-inflammatory, immunostimulant and antihyperglycemic activities in aquatic animals (Taguri et al., 2006;

Bouarab-Chibane et al., 2019; Huu Tinh et al., 2021; Yang et al., 2021; Yuan et al., 2021; Ahmadi et al., 2022; Imperatore et al., 2023).

Tea polyphenols are composed mainly by flavonoids, where catechins amount to 80-90 % of total flavonoids in tea leaves (*Camellia sinensi*). Epigallocatechin gallate (EGCG) is the main catechin in tea leaves, representing 50-80 % of total catechins, and the most biologically active compound among other polyphenols found in tea (Singh et al., 2011; Kim et al., 2014; Yan et al., 2020). The inclusion of tea catechins in aquaculture showed positive results in growth performance, antibacterial, immunostimulant, antiviral and antioxidant activities among others (Thawonsuwan et al., 2010; Wang et al., 2017b; Ji et al., 2018; Zhang et al., 2020; Qian et al., 2021; Zhang et al., 2021; Li et al., 2022). However, tea polyphenols have disadvantages, as they exhibit low stability in biological media; they are easily degraded on physiologically relevant temperature, oxygen concentration and metal ion content on alkaline and neutral pH alike (Krupkova et al., 2016; Xu et al., 2019; Jin et al., 2022). Therefore, oral administration of tea polyphenols leads to low absorption and short half-life (Dang et al., 2013). To overcome these disadvantages, nanoencapsulation has become a strategy to deliver these labile molecules (Dang et al., 2015; Rambaran, 2020; Di Santo et al., 2021).

Nanotechnology has different applications in aquaculture, such as delivery of nutraceutical molecules and vaccines, water purification, pathogen detection, antimicrobial and antiviral activity, and product preservation (Shah and Mraz, 2019; Fajardo et al., 2022). Biopolymer nanocarriers have gained relevance for the delivery of active compounds due to their low toxicity and biodegradability (Esfanjani and Jafari 2016). Zein is a hydrophobic protein found in corn; its low water solubility allows the formation of colloidal nanoparticles in water (Pascoli et al., 2018) and has been widely used for the encapsulation of both hydrophilic and hydrophobic active compounds (Zhang et al., 2014; Nunes et al., 2020; Jin et al., 2022; Zheng et al., 2022). However, zein nanoparticle dispersions display low colloidal stability, a tendency towards aggregation, and precipitation at pH 5-7 (Yuan et al., 2022). Coating zein nanoparticles with biopolymers improves their stability, and the presence of different functional groups facilitates the interaction between nanoparticles and bioactive molecules, thus increasing encapsulation (Yuan et al., 2022). Chitosan and alginate have been used both separately and together to improve the stability of zein nanoparticles (Khan et al., 2019; Carraso-Sandoval et al., 2021).

In this study we have carried out the encapsulation of EGCG and green tea extract in zein nanoparticles stabilized by the layer-by-layer technique with alginate and chitosan. These nanomaterials were characterized and evaluated for their potential to develop activities of interest in aquaculture, such as antioxidant capacity compared to free compounds in solution, and antimicrobial activity against Gram-negative and Gram-positive pathogens of importance in aquaculture.

2. Materials and methods

2.1.Compounds and reactants

Zein from corn, low viscosity sodium alginate and 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH$^{\cdot}$) were obtained from Tokyo Chemical Industries (Tokyo, Japan). Low molecular weight chitosan (135 kDa) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Epigallocatechin gallate 98 % (EGCG) was purchased from Biosynth (Staad, Switzerland). Green tea extract (GTE) with a content of 44 % of EGCG was obtained from Herbadirekt (Wetzlar, Germany). Potassium persulfate 99 % and 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt 98 % (ABTS) were purchased from Glentham life science (Corsham, UK). Glacial acetic acid, absolute ethanol, methanol and culture media were obtained from Labbox (Barcelona, Spain).

2.2.Nanoparticles synthesis

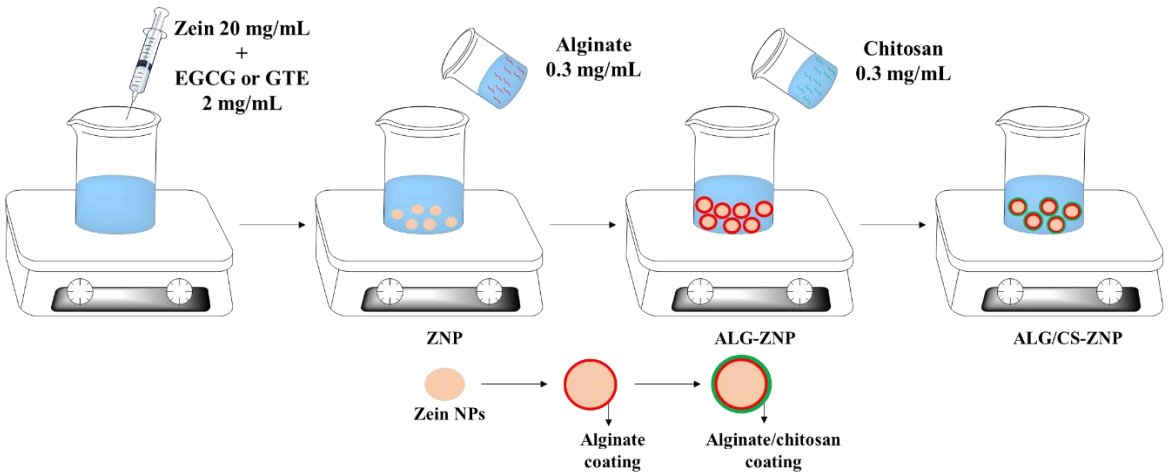

Figure 1. Experimental procedure for zein nanoparticle synthesis and coating with alginate and chitosan.

The preparation of zein nanoparticles (ZNP) have been carried out using the anti-solvent precipitation method based on previous studies with some modifications (Khan et al., 2019; Jin et al., 2022). Briefly, ZNPs were prepared by adding 4 mL of ethanolic (80:20) zein solution (20 mg/mL) into 16 mL of distilled water and magnetically stirred for 30 minutes. Ethanol was removed by rotatory evaporator at 35 ºC for 20 minutes and the loss of volume was compensated with distilled water.

Coating process was carried out by applying the layer-by-layer method based on electrostatic deposition. ZNP dispersion was added to an alginate solution of the same volume prepared at different concentrations (0.2, 0.3, 0.4 and 0.5 mg/mL) and stirred magnetically for 30 minutes. Alginate (ALG) coated zein nanoparticles (ALG-ZNP) were mixed with the same volume of a chitosan (CS) acetic acid solution (1 %) at different concentrations (0.2, 0.3 and 0.4 mg/mL) and stirred magnetically for 30 minutes therefore obtaining alginate/chitosan coated ZNPs (ALG/CS-ZNP). Polyphenol loading was done by adding either EGCG or GTE (2mg/mL) to the starting ethanolic solution of zein, leading to ALG-ZNP-E and ALG/CS-ZNP-E, or ALG-ZNP-T and ALG/CS-ZNP-T particles respectively, depending on whether the synthesis was concluded after the ALG coating step, or the CS shell formation was performed.

2.3.Characterization of nanoparticles

2.3.1. Encapsulation efficiency

Encapsulation efficiency (EE) was obtained based on the method described by Pantoja-Vale et al. (2022). Nanoparticle dispersions were centrifuged at 18,000 g for 20 minutes at 4 ºC (Orto Alresa Biocen 22r, Spain). The polyphenol content of the supernatant was quantified with spectrophotometer (Power Wavex, USA) at 274 nm for both EGCG and green tea extract by applying their calibration curves, y=12.013x + 0.1275, R^2=0,9989 and y=9,246x+0,0903, R^2=0,9999 respectively. Encapsulation efficiency was calculated following the equation:

$$EE\% = \left(1 - \frac{C_{Sup}}{C_R}\right) * 100$$

Where C_{Sup} is the concentration of EGCG or GTE in the supernatant and C_R is the theoretical concentration of EGCG or GTE.

2.3.2. Hydrodynamic size, polydispersity index and ζ-potential

The average hydrodynamic diameter, polydispersity index (PDI) and ζ-potential of the nanoparticles were assessed using a Zetasizer-Nano ZS instrument (Malvern Instruments, Malvern, UK). The nanoparticle dispersions were diluted (1:10) prior analysis and measured in a disposable folded capillary zeta cell (Malvern instruments, Malvern, UK).

2.3.3. Scanning electron microscopy (SEM)

SEM images were obtained with a HITACHI S-3500N instrument with an acceleration voltage of 3 kV. Samples were coated with gold before measurement.

2.3.4. X-ray diffraction (XRD*)*

Nanoparticle dispersions were lyophilized by a Telstar Lyoquest -55 instrument for 48 hours at -50 ºC. XRD measurements between 5-80 (2θ degree) were performed by a D8 Advance Diffractometer (Bruker, Germany) at 50 kV and 50 mA current.

2.3.5. Fourier transform infrared spectroscopy (FTIR)

FTIR measurements used a Bruker Vertex 70 instrument (32 scans, 4 cm^{-1} resolution) with the KBr pellet technique and compressed to tablets. 4000-400 cm^{-1} wavenumber region transmittance was recorded.

2.4.Antibacterial assay

Lyophiles of *Vibrio anguillarum* (CECT 522), *Vibrio alginolyticus* (CECT 436), *Photobacterium damselae* (CECT 5122), *Pseudomonas anguilliseptica* (CECT 899) and *Streptoccocus iniae* (CECT 7363) were obtained from the Spanish Type Culture Collection of the University of Valencia (Valencia, Spain). Lyophiles were recovered and maintained following the instruction provided by CECT. The inocula of all bacteria were grown in TSB 1 % NaCl at 37 ºC for *S. iniae*, 30 ºC for *V. anguillarum* and *V. alginolyticus* and 25 ºC for *P. anguilliseptica* and *P. damselae*. The concentration of each inoculum was adjusted to 0.5 OD at 600 nm. The assay was prepared by adding 0.5 mL of standardized inoculum and 0.5 mL of freshly prepared nanoparticle dispersion into 4 mL of TSB 1 % NaCl in a test tube. Oxytetracycline (10 µg/mL) was used as positive control. Free EGCG and GTE (1 mg/mL) were also assayed. Blank tubes were prepared adding 0.5 mL of nanoparticle dispersions into 4.5 mL of culture medium. Three tubes were prepared for each sample.

After 48 hours of incubation in a thermostatized agitated water bath, sample absorbance was recorded on 600 nm. The calculation of inhibitory rate (IR%) was conducted following the next equation:

$$IR\ (\%) = \frac{(I - I_B) - (A_S - A_{BS})}{(I - I_B)} * 100$$

Where A_S is the absorbance of inoculum with nanoparticle dispersion or active compound, A_{BS} is the absorbance of its respective blank, I is the absorbance of inoculum and I_B is the absorbance of the culture medium.

2.5.Antioxidant activity

To examine the antioxidant activity of prepared nanoparticles DPPH and ABTS tests have been carried out following the methods described by Xiao et al. (2020).

2.6.Statistical analysis

Analysis was performed on triplicates using ANOVA one way test, the results are expressed as mean ± SE. Subsequently, a comparison of means (Tukey's HSD test) has been carried out, significance level was set as $P<0.05$. All calculations have been carried out using the IBM SPSS Statistics 28 software (2022).

3. Results and discussion

3.1.Nanoparticles characterization

3.1.1. Particle size, polydispersity index and ζ-potential

Nanoparticle dispersions have been characterized for their hydrodynamic size, polydispersity index and ζ-potential. Table 1 displays effects on ZNP when they were coated with alginate at different concentrations. ZNP with no coating were smaller than ALG-ZNP at any concentration of alginate used.

Table 1. Size, polydispersity index and ζ-potential of zein nanoparticles coated with different alginate concentration (ALG-ZNP).

ALG (mg/mL)	Hydrodynamic size (nm)	PDI	ZP (mV)
0	113.90 ± 1.37^{a}	0.161 ± 0.003	+20.63 ± 0.86^{a}
0.2	138.80 ± 1.47^{bc}	0.172 ± 0.013^{a}	-11.27 ± 0.40^{b}
0.3	142.06 ± 1.22^{cd}	0.182 ± 0.014^{ab}	-33.63 ± 0.83^{c}
0.4	145.97 ± 1.95^{b}	0.195 ± 0.004^{a}	-42.57 ± 0.83^{d}
0.5	152.80 ± 3.05^{d}	0.234 ± 0.004^{bc}	-53.90 ± 0.44^{e}

Means (± SE) from 3 individual measurements. The same letter within a row is not significantly different from one another ($P < 0.05$). PDI: polydispersity index; ZP: ζ-potential.

Hydrodynamic size of ALG-ZNP increased as the concentration of ALG in solution raised. Similar results were obtained for alginate/zein nanoparticles by Carrasco-Sandoval et al. (2021). A possible explanation is that size increased as more layers of ALG were deposited on the nanoparticle surface (Jiang et al., 2021). PDI displays similar trend as showed for particle size, i.e., the increase of ALG concentration raised PDI, which means a reduction in the homogeneity of the dispersion (Raval et al., 2019). Z-potential of ZNP trended from positive to negative when the negatively charged ALG was introduced to the system, thereby stabilizing the dispersion of ZNP by increasing its absolute charge value in higher concentrations. After addition of 0.2 mg/mL ALG, the measured ζ-potential was -11.27 mV and precipitation was observed after some minutes of stirring. This can be due to the neutralization of the highly positive charge of zein and the negative charge of alginate, resulting in a low stability dispersion at this concentration (Khan et al., 2019). After the evaluation of this results, ALG concentration of 0.3 mg/mL was selected as it gave smaller particles size and PDI, while ζ-potential absolute value was >30 mV, denoting the dispersion as electrostatically stable (Samimi et al., 2019).

Table 2 displays results of hydrodynamic size, PDI and ζ-potential of ALG-ZNP coated by chitosan at different concentrations. Size was higher for ALG/CS-ZNP dispersions than ALG-ZNP. No significant differences could be observed for PDI and ζ-potential between 0.2 and 0.3 mg/mL chitosan solution. When concentration of chitosan solution reached 0.4 mg/mL, PDI and ζ-potential increased significantly due to an excess of chitosan on the dispersion. On account of these results, the formulation used for the encapsulation of EGCG, and GTE was 0.3 mg/mL for both alginate and chitosan solutions.

Table 2. Hydrodynamic size, polydispersity index (PDI) and ζ-potential (ZP) of zein/alginate nanoparticles coated with different chitosan concentration (ALG/CS-ZNP).

CS (mg/mL)	Hydrodynamic size (nm)	PDI	ZP (mV)
0.2	212.60 ± 4.71^{b}	0.196 ± 0.005^{a}	42.10 ± 2.16^{a}
0.3	199.60 ± 2.55^{a}	0.198 ± 0.011^{a}	40.27 ± 1.39^{a}
0.4	271.83 ± 1.07^{c}	0.303 ± 0.024^{b}	52.10 ± 1.18^{b}

Means (± SE) from 3 individual measurements. The same letter within a row is not significantly different from one another ($P < 0.05$). PDI: polydispersity index; ZP: ζ-potential.

Regarding encapsulation efficiency of EGCG and GTE of ZNPs, levels of 45 % and 58 % were achieved respectively (data not shown).

After the coating with ALG an increase could be observed (table 3), reaching 75 % and 80 % of encapsulation, respectively. Upon the addition of chitosan into the system, encapsulation increased up to 82 % and 85 % respectively. These results are similar to those obtained by other studies (Jin et al., 2022; Liang et al., 2017) based on EGCG encapsulation in ZNP, although with different stabilization compounds. DLS analysis (table 3) of EGCG and GTE encapsulated on ZNP coated with ALG and CS displayed higher PDI values for all formulations, although hydrodynamic size increased for ALG/CS-ZNP-E and ALG/CS-ZNP-T compared to blank nanoparticles. Z-potential did not vary substantially, and it can therefore be concluded that the addition of EGCG or GTE did not alter the colloidal properties of the particles.

Table 3. Hydrodynamic size, polydispersity index (PDI), ζ-potential (ZP) and encapsulation efficiency (EE) of different formulations

Formulation	Hydrodynamic size (nm)	PDI	ZP (mV)	EE %
ALG-ZNP-E	143.73 ± 0.68^{a}	0.245 ± 0.005^{b}	-31.47 ± 0.12^{a}	75.24 ± 0.97^{a}
ALG-ZNP-T	144.03 ± 0.18^{a}	0.202 ± 0.002^{a}	-30.73 ± 0.49^{a}	80.13 ± 1.13^{b}
ALG/CS-ZNP-E	238.90 ± 0.65^{b}	0.297 ± 0.001^{d}	42.93 ± 0.28^{b}	82.04 ± 1.15^{b}
ALG/CS-ZNP-T	266.47 ± 0.66^{c}	0.270 ± 0.007^{c}	41.97 ± 0.38^{b}	85.14 ± 0.49^{c}

Means (± SE) from 3 individual measurements. The same letter within a row is not significantly different from one another ($P < 0.05$). PDI: polydispersity index; ZP: ζ-potential; EE: encapsulation efficiency.

3.1.2. Morphology characterization

Figure 2 shows SEM pictures of the three nanoparticle structures prepared throughout our experiments. The image of ZNPs (figure 2A) displays spherical structures with smooth surfaces, although small adhesion of the particles can be observed. This can be due to the film-forming capacity of zein during solvent evaporation (Jiang et al., 2021). Particles had an average size of 30 nm, which is significantly smaller than the size observed in DLS measurements. The difference can be mainly due to the dehydration and the consequent shrinking of the particles. In addition, particles within aggregates can be visually distinguished, whereas DLS measurements cannot distinguish between well dispersed single particles and compact aggregates. The ALG-ZNP image (figure 2B) also displayed spherically shaped particles, but size was larger than that of ZNP. The mean size of particles was 92 nm, although bigger particles were also visible. The addition of CS layer to nanoparticles did not change the size observed by SEM regarding ALG-ZNP (figure 2). However, increased adhesion can be observed between particles, as well as a layer in which the particles are embedded. These results are similar to those obtained in

studies analyzing the same materials (Khan et al., 2019; Pauluk et al., 2019; Khan et al 2021). Lin et al. (2020) obtained similar results for carboxymethyl chitosan coated zein nanoparticles. The film-like structure could improve the re-dispersibility of the nanoparticles after the drying process, and the release rate of encapsulated compound.

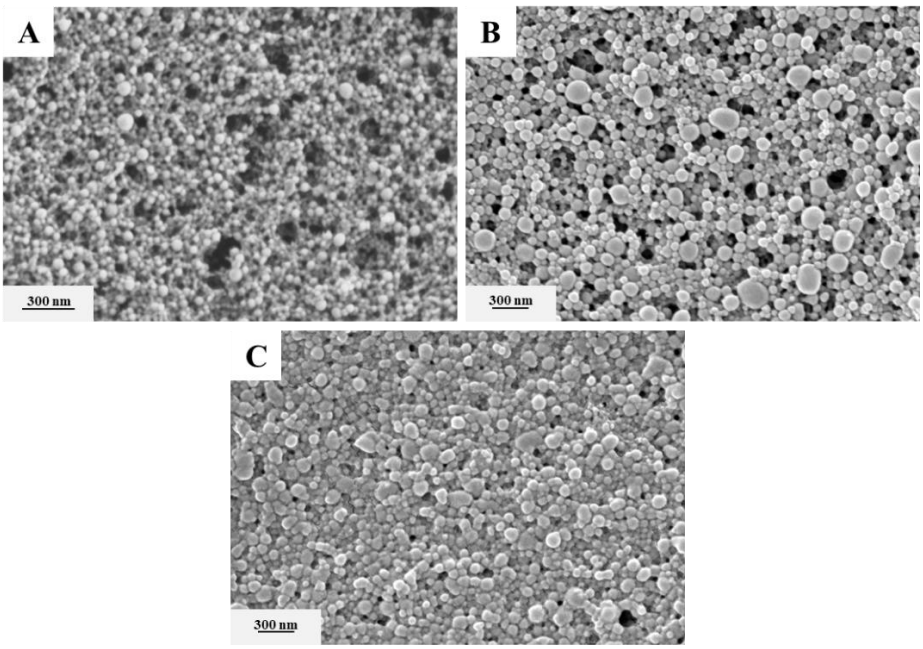

Figure 2. Scanning electron microscope images of ZNP (A), ALG-ZNP(B) and ALG/CS-ZNP.

3.1.3. X-ray diffraction (XRD) analysis

The X-ray diffractogram of the starting materials and the nanoparticle powders can be observed on figure 3, where zein displays two wide characteristic absorption peaks at 9.5º and 20º related to the α-helix structure within zein (Jiang et al., 2021). The sodium alginate diffractogram showed two broad peaks at 13.4º and 21.8º, while chitosan displayed two peaks at 10º and 20º. This showed that both polysaccharides had semi-crystalline characteristics, which is consistent with previous studies (Sundarrajan et al., 2012; Bhagyaraj and Krupa, 2020; Ju et al., 2020). EGCG has characteristic sharp peaks at 15.5º, 17º, 20.5º, 21.4º, 24.4º, 25.8º and several small sharp peaks until 50º. This demonstrated the crystalline properties of EGCG (Fang et al., 2019; Xie et al., 2021; Zhao et al., 2022). Green tea extract showed a broad peak from 10º to 35º, and some sharp peaks can be observed at 6º, 19º and 24.3º.

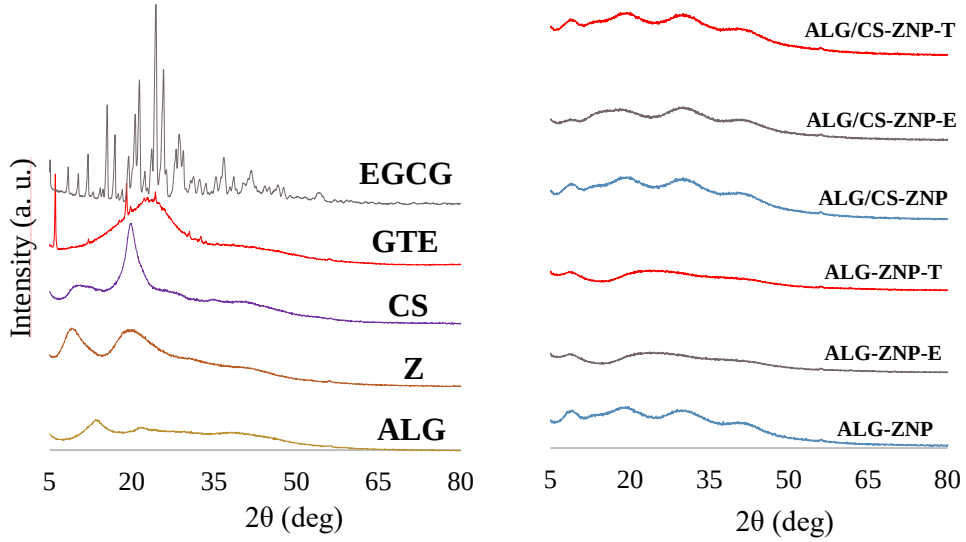

Figure 3. X-ray diffractograms of starting materials and nanoparticle powders.

The X-ray diffractograms of ALG/CS-ZNP-E and ALG/CS-ZNP-T did not display the characteristic sharp peaks present in EGCG or the green tea extract, indicating that the encapsulated compounds did not exist in crystalline form, and that EGCG and green tea extract may be encapsulated within the nanoparticles (Gao et al., 2021; Jin et al., 2022). It also can be noted that the characteristic peaks of the polymers decreased in intensity, probably due to the interactions between the polymers themselves and the encapsulated compounds (Khan et al., 2019; Jin et al., 2022). Supplementary data (figure S1) displays the physical mixture of both ALG/CS-ZNP-E and ALG/CS-ZNP-T. In the physical mixture containing EGCG, some of the sharp peaks denoted to the crystalline polyphenol can be seen with decreased intensity due to its low concentration within the mixture. For GTE, the physical mixture the 6º peak can be slightly observed in the diffractogram. As these crystalline peaks are only present in the mixtures, but not in the nanostructures, in can be concluded that encapsulation was successful.

3.1.4. FTIR analyses

Figure 4 displays FTIR spectrum of pure EGCG and GTE. The EGCG spectra displays characteristic peaks at 3474 and 3347 cm^{-1} (O-H stretching), 1689 cm^{-1} (C=O stretching), 1612, 1533 and 1446 cm^{-1} (C=C, aromatic stretching), 1341 cm^{-1} (O-H bending), 1215 cm^{-1} (C-O stretching) and 1144 cm^{-1} (C-O stretching from tetrahydropyran ring), 1092 and 1008 cm^{-1} (aromatic ring stretching) (Robb et al., 2002; Billes et al., 2007; Wang et al., 2019). The GTE spectra was similar to that of EGCG, with slight shifts on some peaks

due to the presence of other components in lower concentration, indicating that a high concentration of EGCG was found in this commercial extract.

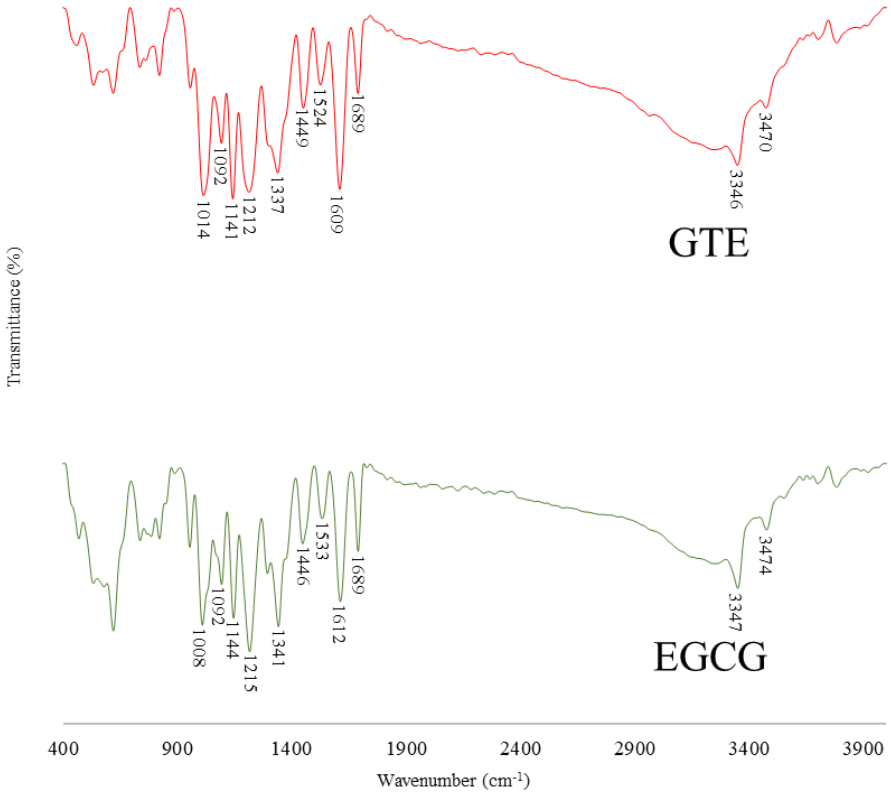

Figure 4. FTIR spectrograms of EGCG (A) and GTE (B).

Figure 5 shows FTIR spectra of ALG-ZNP, ALG-ZNP-E and ALG-ZNP-T. All nanoparticle formulations had two characteristics peaks of zein at 1653 and 1536 cm^{1}, from amide I (C=O stretching) and amide II (N-H bending), respectively (Jin et al., 2022). ALG-ZNP-E and ALG-ZNP-T displayed a peak around 1144 cm^{-1} derived from the C-O stretching of tetrahydropyran ring. The rest of the EGCG or GTE peaks did not appear, while in the physical mixture of EGCG and GTE with the components of nanoparticles (Figure S2) more peaks could be observed, which suggests that compounds were encapsulated. Figure S3 displays the spectra of ALG/CS-ZNP, ALG/CS-ZNP-E and ALG/CS-ZNP-T FTIR. Slight peak shifts could be observed upon the addition of chitosan to the mixture of alginate coated nanoparticles. This information provides us with further evidence of the encapsulation of bioactive compounds in the nanoparticles.

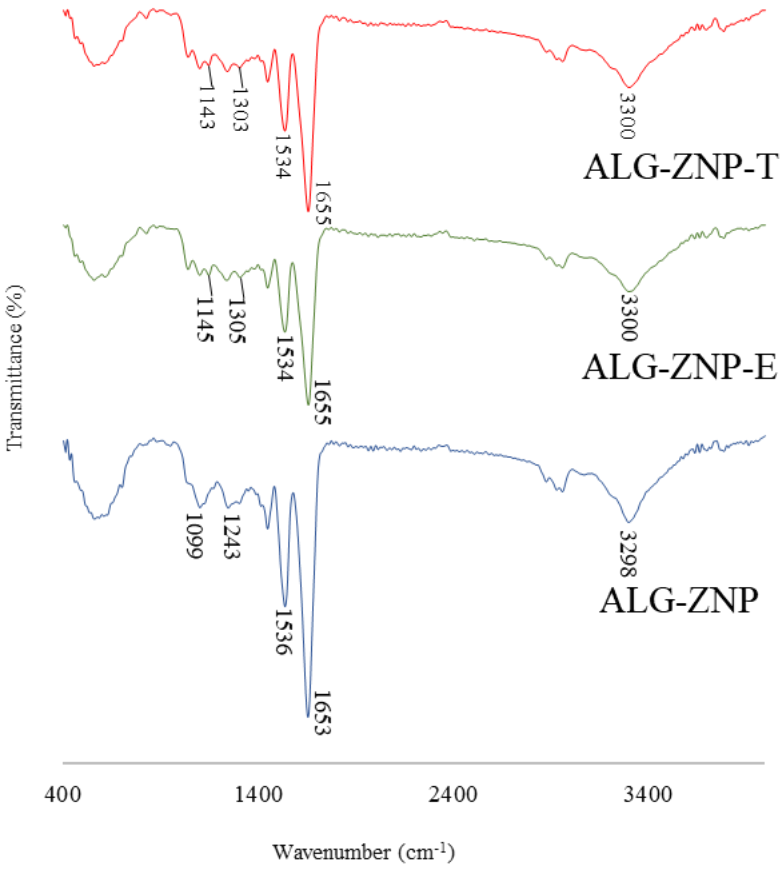

Figure 5. FTIR spectrograms of ALG-ZNP blank nanoparticles and ALG-ZNP-E and -T nanoparticles.

3.2.Antioxidant activity

As stated before, catechins have a great number of biological activities with several benefits to aquatic organisms. A large part of these beneficial biological activities stem from their high antioxidant power. Catechins can exhibit antioxidant power directly, by scavenging free radicals, or indirectly, through the activation of antioxidant enzymes, EGCG being the one with the highest antioxidant power (Berantoniene and Kopustinskiene, 2018). However, their absorption is limited *in vivo* due to their labile nature (Kim et al., 2014), for which reason we propose its encapsulated use.

Free radicals are molecular species with an unpaired electron in their outer atomic orbital. These molecules, due to their high instability, can react with different cellular components (DNA, lipids, and proteins) causing cellular damage which may lead to organ failure (Lobo et al., 2010). Therefore, it is important to test the free radical scavenging potential of nanoparticles and encapsulated compounds. The antioxidant power *in vitro* of

encapsulated EGCG and GTE have been carried out and compared to free substances. Figure 6 shows the SC50 of DPPH and ABTS assays of the different nanoparticles synthesized compared to free substances.

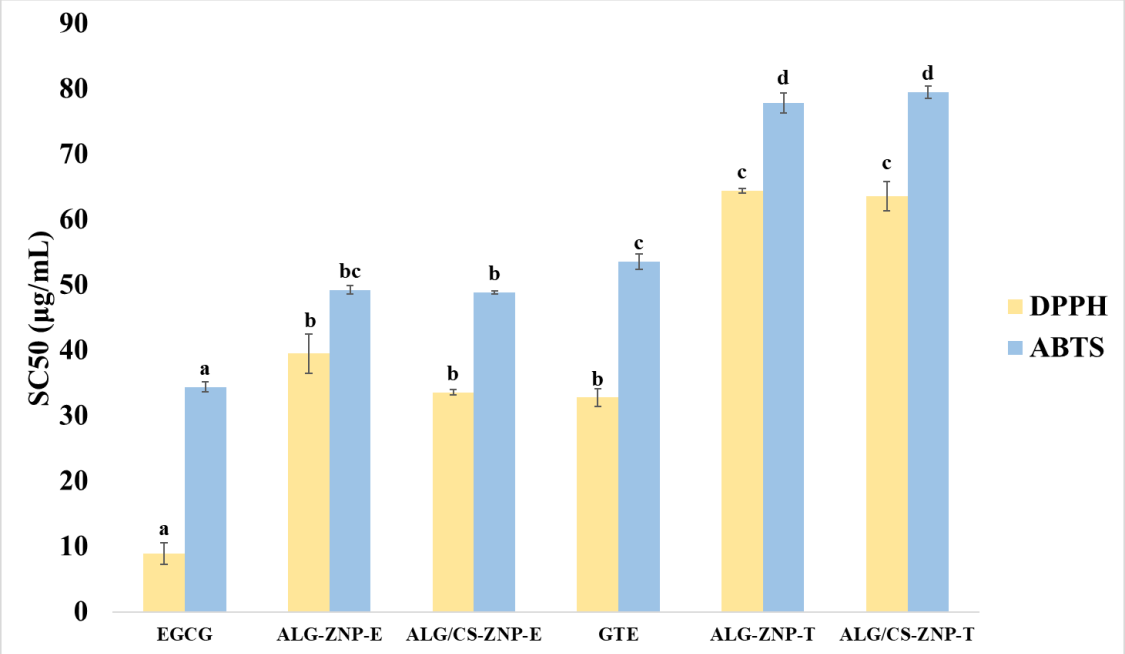

Figure 6. Antioxidant assays (DPPH and ABTS) tested on nanoparticles and free EGCG and GTE. Different letters referring to the same antioxidant compound indicate the existence of significant differences for the activity shown by the different types of nanoparticles tested ($P<0.05$).

Similar trends can be observed for the two assays tested. In general, the minimum concentration to scavenge 50 % of free radicals (SC50) is higher when the compounds were encapsulated. This increase in the SC50 and therefore, decrease in the antioxidant power, might be due to the fact that when the substances are encapsulated, they may be forming hydrogen bonds in addition to other types of interactions with the encapsulation matrix (Li et al., 2009). These interactions reduce the antioxidant capacity of polyphenols (Gulcin, 2020).

The SC50 of EGCG was lower than that of GTE. EGCG accounts for 45 % of GTE composition, while the rest being mostly other catechins which have less antioxidant power. The DPPH showed an SC50 for EGCG and GTE of 8.92 and 32.79 µg/mL, respectively. After encapsulation in zein and subsequent coating with alginate, the SC50 of ALG-ZNP-E and ALG-ZNP-T increased to 39.54 and 64.43 µg/mL. Coating with chitosan caused a decrease in SC50 for ALG/CS-ZNP-E (33.58 µg/mL) with respect to

ALG-ZNP-E, although it did not show significant differences. For ALG/CS-ZNP-T, the coating did not significantly affect the SC50 (63.59 μg/mL).

In the ABTS radical assay for EGCG and GTE, the SC50 increased compared to the DPPH assay, 34.42 and 53.56 μg/mL respectively. However, the response profile obtained was similar to that described previously. Thus, the SC50 increased for the encapsulated compounds with respect to the free substances. ALG-ZNP-E and ALG-ZNP-T needed 49.23 and 77.86 μg/mL to scavenge 50% of the ABTS radicals. The addition of chitosan did not result in a worsening of the antioxidant power of the nanoparticles. ALG/CS-ZNP-E and ALG/CS-ZNP-T showed an SC50 of 48.87 and 79.54 μg/mL. According to Osman et al. (2006), the oxidation process experienced by the polyphenolic compounds takes place in different positions depending on the method used, which could explain the differences observed in the results obtained for each of the protocols applied. Additionally, this could imply that the interactions between the nanoparticle constituents and the active substances they encapsulate occur at specific positions. The decrease in the availability of these specific positions would therefore affect the antioxidant activity assay differently depending on the substrate used.

These results show that the antioxidant power of the catechins present in the nanoparticles is diminished due to encapsulation. However, considering that encapsulation confers protection to the active materials against hostile conditions that they would face in their use in the aquaculture sector, such as those inherent to digestive processes, the antioxidant power still retained by the nanoparticles can be considered adequate (Khan et al., 2019; Pauluk et al., 2019; Liang et al., 2021).

3.3.Antimicrobial activity assay

Figure 7 shows the results obtained in relation to the antimicrobial activity shown by the different active ingredients tested and the different formats in which they were tested.

Considering the results from a global perspective and with the type of material tested as a unifying criterion, the antimicrobial activity exhibited by the nanoparticles with the presence of chitosan stood out, the only ones that, with inhibition levels between 56 % and 60 %, were close to the values reached by the antibiotic (85 %), established as a positive control. The rest of the tested formats did not exceed 40 % inhibition in any case, although the nanoparticles with alginate and encapsulated product, both EGCG and tea extract, did not generate significant differences, for the most part, with the previously

mentioned chitosan formats. The lowest inhibitory capacity was observed for alginate nanoparticles without active product, as well as for the active products themselves in free format, with percentages ranging from 16 % to 21 %. Encapsulation of materials with antimicrobial activity in mixed alginate-chitosan nanoparticles has been revealed as a suitable practice to increase the efficiency of such materials (Zimet et al., 2018; Yoncheva et al., 2021). However, and based on the results obtained in the present study, with the majority absence of significant differences between the inhibitory capacity of empty nanoparticles and that of nanoparticles with active materials, as reported in other works (Kadhum and Zaidan, 2020), it is worth asking whether the activity of such materials is really increased. In the case of alginate nanoparticles, there does seem to be such an effect, especially in the case of EGCG, the only case in which there was a significant increase in the percentage of growth inhibition, both with respect to empty nanocapsules and free EGCG. On the contrary, in the case of nanoparticles with the additional presence of chitosan, although the inhibitory capacity was higher in those that included EGCG, the increase was not sufficiently intense to generate a statistical difference. It would be attributable in this case, therefore, mostly to the components of the nanoparticles and, in particular, to the chitosan. Friedman et al. (2013) also observed a significant inhibitory activity of empty nanoparticles, attributed to the affectation of the lipid fraction of the cytoplasmic membrane. Additionally, and similar to that described in the present work, chitosan was shown to be mainly responsible for the microbial growth reducing potential. It follows, under these conditions, that not all the amino groups present in chitosan, which confer the polymer with positive charge, are counteracted by the negative charge of the carboxylic acids associated with alginate, thus leaving potential to interact with the cell cytoplasmic membrane through their negative charges (Al-Gethami and Al-Qasmi, 2021). However, it is not advisable to generalize these conclusions, since the results associated with each of the components may vary depending on the microorganism used in the assay (Paiva et al., 2020; Adadpoor et al., 2021).

The difference in response offered by different microorganisms can be seen in Figure 7B. This figure shows *Vibrio anguillarum* as the species least sensitive overall to the action of the different formats tested, with an average percentage of growth inhibition slightly higher than 18 %, while *Photobacterium damselae* was the most affected (56.12 %), although with little difference with respect to *Pseudomonas anguilliseptica* (51.89 %). Between the two extremes were *Streptococcus iniae* and *Vibrio alginolyticus*, statistically

related to the two previous groups. The existence of different responses to the same compound is not only common among different species (Ignasmithu et al., 2019), but also among strains of the same species (Siriphap et al., 2022). The mechanisms through which EGCG and other green tea polyphenols act are diverse (Reygaert, 2018), as are the pathogenic strategies adopted by different strains and the specific composition of their cellular structures, so varied responses are to be expected in assays of this nature. Generally speaking, Gram-positive species are considered to be more sensitive to polyphenols (Zhang et al., 2015). With regard to EGCG. and in nanoparticle format, a higher resistance of Gram-negative bacteria is postulated as a consequence of the existence of the outer membrane and lipopolysaccharide, which limits the potential of nanoparticles to bind to the peptidoglycan layer (Zhao et al., 2022). The sensitivity exhibited by certain Gram-negative species would be more related to the production of oxidative damage (Cui et al., 2012). In the case of *V. alginolyticus*, the ability of phenolic compounds to reduce its biofilm-forming capacity has been demonstrated, among other factors, by affecting the biosynthetic potential of polysaccharides, in addition to altering the permeability of the cytoplasmic membrane (Liu et al., 2021). In the case of *P. damselae*, bacteria that usually register higher levels of sensitivity to polyphenolic compounds than other fish pathogens (Bulfon et al., 2014), as observed in the present study, the possible impairment of biofilm-forming capacity as well as motility potential has also been postulated (Bautista-Rosales et al., 2022). In the case of *V. anguillarum*, the species for which the lowest degree of inhibition was recorded, compounds of a different nature, such as antimicrobial peptides or polyunsaturated fatty acids, seem to be more effective in its control (Citarasu, 2012).

Figure 7C shows the individualized response of each microorganism for each of the formats tested. The levels of growth inhibition obtained in each case confirm what was previously mentioned regarding the variability detected depending on the microorganism studied. Thus, *S. iniae* and *P. damselae* showed a differentially significant sensitivity between nanocapsules with and without chitosan incorporation. In both cases, the levels of growth inhibition experienced by both bacteria were clearly higher for the former, and the Gram-positive species even showed a higher degree of cell development in the media with nanocapsules without chitosan than in the control free of antimicrobial compounds. The greater efficacy of nanoparticles with chitosan in this case may be due to the interactions between the positive charges provided by this polymer and the negative

charges of the teichoic acids present in the Gram-positive cell wall (Kaur et al., 2020), thus favoring the altering potential on the cytoplasmic membrane and, therefore, its antimicrobial capacity (Raafat et al., 2008). Regarding *P. damselae*, and although most studies point to a lower sensitivity of Gram-negative species to chitosan (Li and Zhuang, 2020), this polymer has also shown interesting levels of inhibitory activity in relation to certain bacteria of this group. In the most sensitive species, it is postulated that its greater degree of affectation could be related to a higher hydrophilic character, which would favor the access of the compound to the cellular interior in greater proportion (Chang et al, 2004). In contrast, *V. alginolyticus* and *V. anguillarum* were affected to a greater extent by nanoparticles consisting only of alginate, especially those carrying EGCG. In this case, the inhibition levels achieved led to significant differences with all the chitosan nanoparticles, in the case of the first bacterium, while in the case of the second, the significance was limited to the chitosan nanocapsules with green tea extract. The last of the species tested, *P. anguilliseptica*, showed a less defined pattern, with inhibition values close for all the formats, which meant that few of them differed significantly from each other. As previously discussed, chitosan nanoparticles seem to show lower efficiency on Gram-negative species, although the results reflected in the existing literature are somewhat contradictory (Chandrasekaran et al., 2020). The great variability that exists in terms of the conditions under which the studies are carried out, especially with regard to the presence of additional materials in the nanoparticles, makes it difficult to obtain homogeneous results and, therefore, unique conclusions. However, and depending on the mechanisms of action mostly recognized for chitosan (interaction with different negatively charged structures present in cell envelopes), the specific molecular architecture associated with each species can determine and condition different degrees of sensitivity to chitosan nanoparticles (Duan et al., 2019). Regarding the active substances tested, EGCG and green tea extract, a similar pattern was observed for all bacteria, so that no statistically significant levels of inhibition were found between nanocapsules of the same type carrying different antimicrobial substances. This last result is quite positive from an economic perspective, given the great difference in cost between a natural extract and a pure compound from this extract, and the importance that this factor reaches in industrial processes.

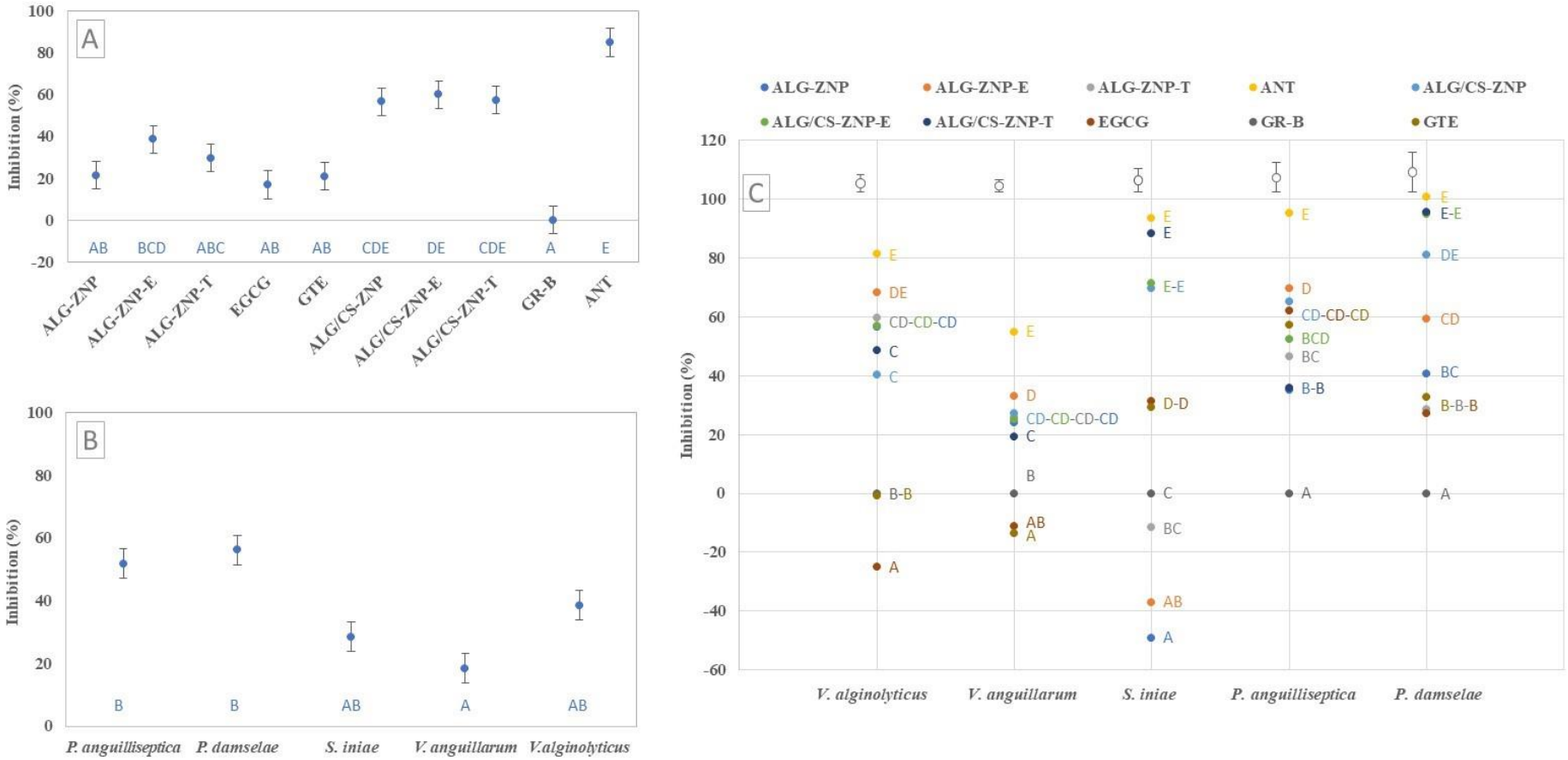

Figure 7. Antimicrobial activity associated with the different formats of active substances and nanoparticles tested. A) Overall activity as a function of the type of substance. B) Global activity as a function of the target microorganism. C) Individualized activity for each of the bacteria tested. In all cases the mean values and the corresponding standard error are shown. The letters associated with each value reflect the homogeneity groups generated by the Tukey's HSD test, with global character in cases A and B, and associated to bacterial species, in case C. ALG-ZNP: alginate NP; ALG-ZNP-E: alginate NP with EGCG; ALG-ZNP-T: alginate NP with green tea extract; GTE: green tea extract; ALG/CS-ZNP: alginate/chitosan NP; ALG/CS-ZNP-E: alginate/chitosan NP with EGCG; ALG/CS-ZNP-T: alginate/chitosan NP with green tea extract; GR-B: growth control; ANT: antibiotic.

4. Conclusions

The current restrictions on the use of antibiotics in the aquaculture sector have favored in recent years the implementation of alternative strategies to ensure the welfare and health of fish. Within the framework of such strategies, polyphenolic compounds have been proposed for the preventive treatment of specimens grown in aquaculture, although it is necessary to develop application protocols to solve the problems related to their sensitivity to adverse conditions in the digestive tract. The present work proposes the encapsulation of EGCG and green tea extract and the evaluation of such formats in relation to their antioxidant and antimicrobial activity. The synthesis has been carried out by stabilizing the zein by a layer-by-layer method with alginate and chitosan. The particles were characterized by DLS, FTIR, SEM and XRD. These nanoparticles displayed spherical shape and a maximum size of 260 nm. The encapsulation of EGCG or GTE was higher than 80 % in most cases. The results obtained show that these formulations were able to maintain a large part of their antioxidant activity with respect to free substances. In relation to antimicrobial activity, the growth inhibition potential showed dependence on the pathogenic species under study, although nanoparticles with chitosan appear to be more effective, in some cases even reaching levels of inhibition close to those of the antibiotic selected as a positive control. These results open new paths for the use of these nanoparticles in disease control and/or a possible synergy with the antibiotic, thus reducing the amount needed. Moreover, the encapsulation protects the substance against degradation and can be stored either as an isolated product or as part of the formulation in aquaculture diets. Further studies should be carried out on the release of the substances in the digestive process, as well as the stability in dispersion or as dry material.

5. Acknowledgements

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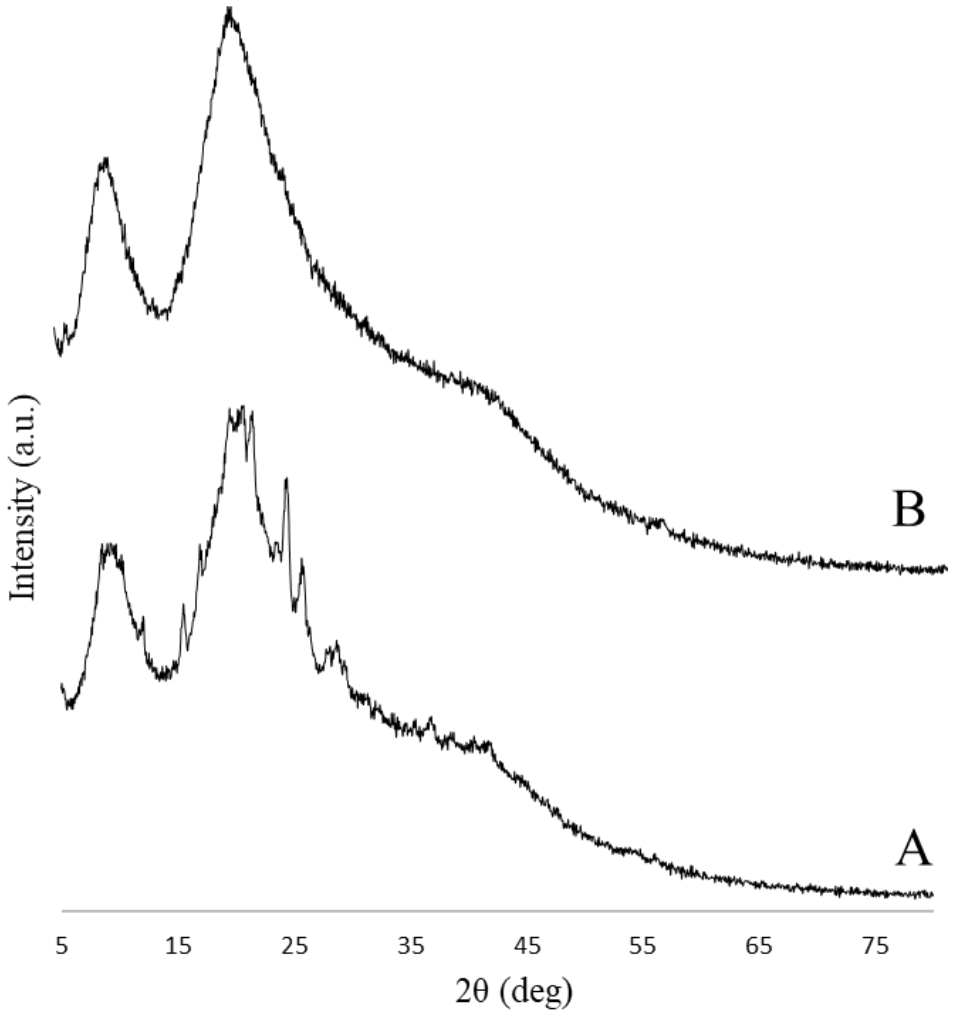
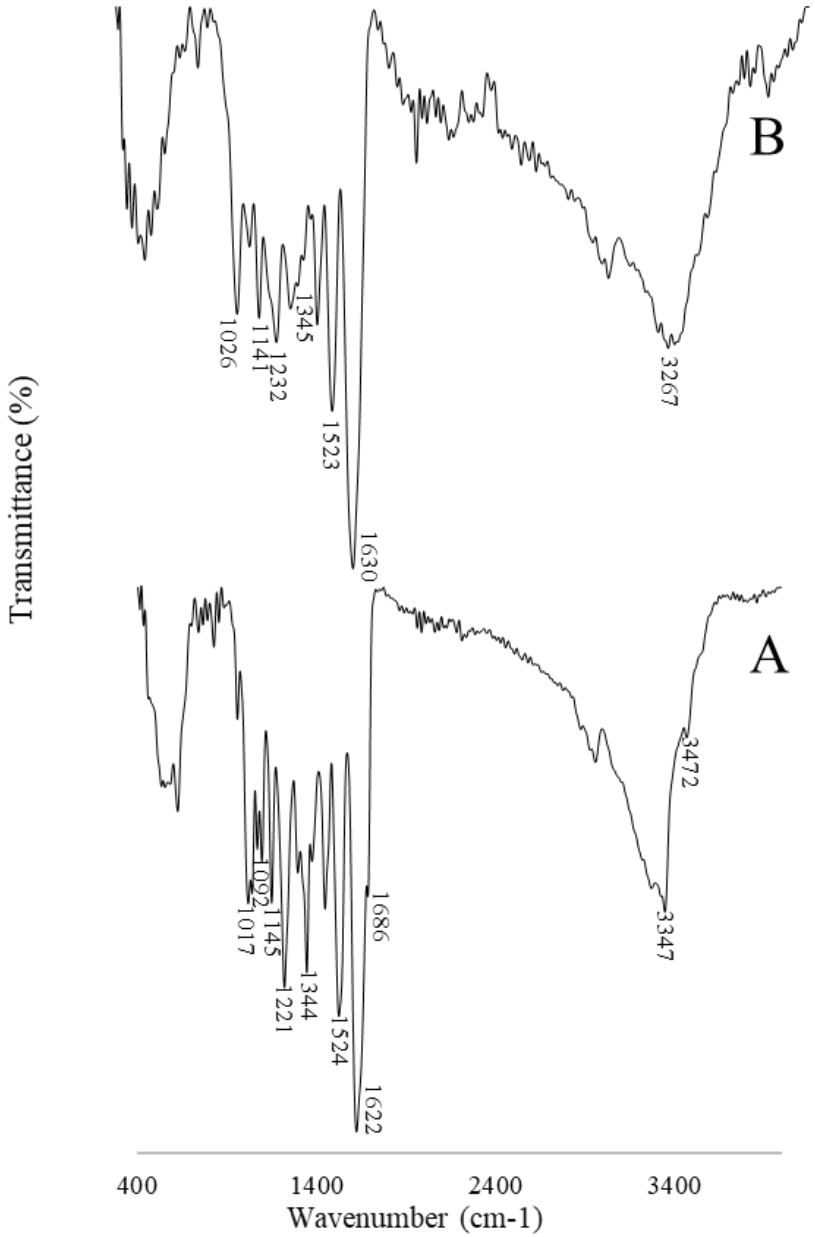
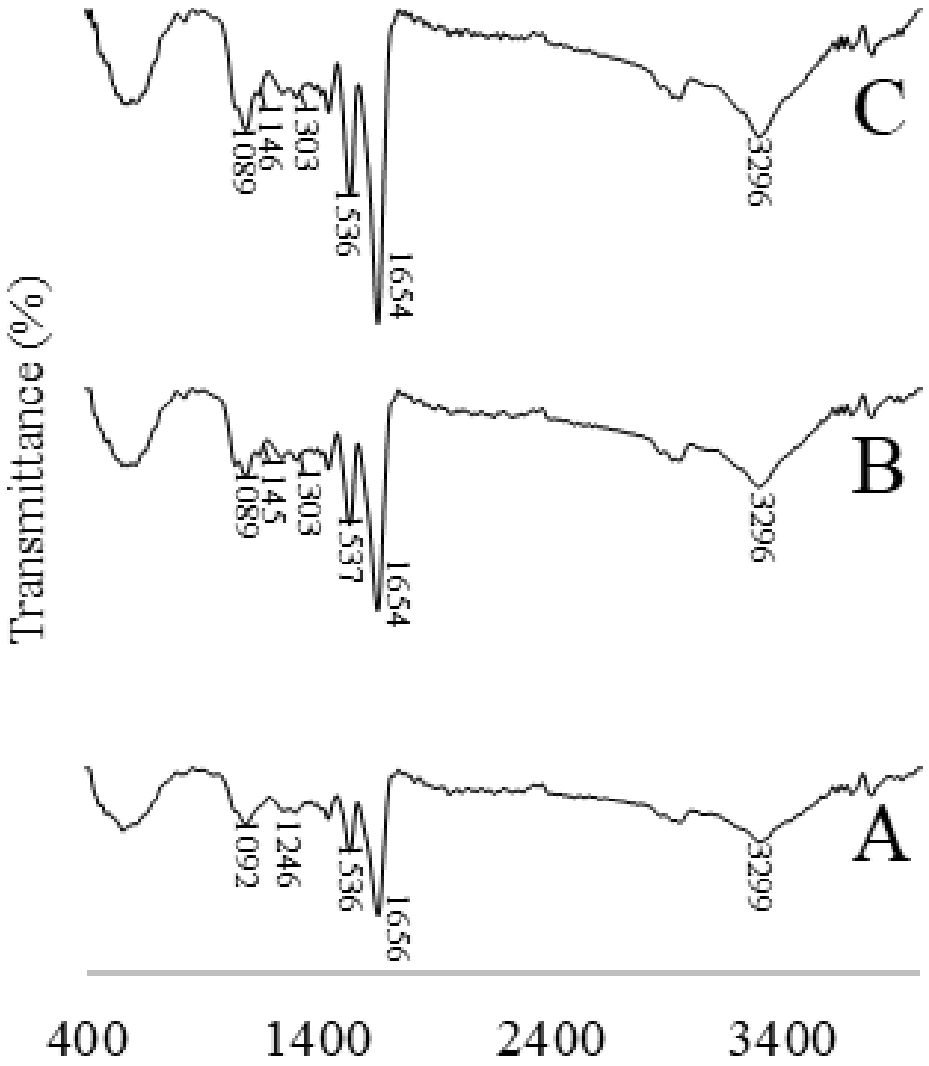
6. Supplementary material



S1. XRD diffractogram of physical mixture of EGCG, alginate, chitosan and zein (A) and GTE, alginate, chitosan and zein (B).



S2. FTIR spectrum of physical mixture of EGCG, alginate, chitosan and zein (A) and GTE, alginate, chitosan and zein (B).



S3. FTIR spectrum of ALG/CS-ZNP (A), ALG/CS-ZNP-E (B) and ALG/CS-ZNP-T (C)

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DISCUSIÓN GENERAL

Para entender la relevancia de esta tesis hay que poner en contexto la importancia que tiene la industria acuícola en la alimentación humana. La acuicultura se ha propuesto como una de las fuentes proteicas más relevantes en los próximos años, debido a que permite aumentar el acceso a un producto de alta calidad nutritiva. Su crecimiento se ha estimado en un 30 % desde la década de los noventa, y se prevé que siga aumentando hasta un 60 % para el 2030 (World Bank, 2013). Sin embargo, este crecimiento no está exento de controversias. Actualmente, la acuicultura enfrenta serios problemas de sostenibilidad, tales como la eutrofización, el empleo de harina y aceite de pescado, el uso generalizado de antibióticos, la destrucción de ecosistemas naturales y la contaminación de agua de consumo humano, entre otros (Diana, 2009; Martinez-Porchas y Martinez-Cordova, 2012; Thurlow et al., 2019; Carballeira Braña et al., 2021), que se ven agravados por efecto del cambio climático (Maulu et al., 2021). Tal como se ha comentado en la introducción, el uso de la harina de pescado afecta a la cadena trófica del ecosistema donde se capturan los peces destinados a la elaboración del producto. Esto trae como consecuencia una reducción del alimento para animales de niveles tróficos superiores (Alder et al., 2008; Pikitch et al., 2012). Por otro lado, no hay que obviar que, debido a la sobrepesca y los efectos del cambio climático, se ha reducido la población de las especies destinadas a la elaboración de harina de pescado (Jannathulla et al., 2019; Canales et al., 2020; Shannon y Waller, 2021). La producción de harina de pescado no puede mantener el ritmo de demanda que requiere la acuicultura, por lo que su precio ha ido en aumento año tras año (Jannathulla et al., 2019). La dependencia de este sector de la harina de pescado trae consigo un aumento de precio en el producto que adquiere el consumidor, lo que limita su acceso. Junto a la harina de pescado, se usa habitualmente harina de soja como fuente proteica en dietas de acuicultura. Aunque su precio es menor, trae consigo otra serie de problemas ambientales y nutricionales (Chou et al., 2004; Sánchez-Muros et al., 2014; Gu et al., 2016).

El futuro cercano que se plantea prevé que la acuicultura logre aumentar su producción para satisfacer las demandas de proteína de una población creciente, y se sitúe como una industria más sostenible. Estas proyecciones pasan, entre otras cosas, por encontrar alternativas proteicas a la harina de pescado.

En los últimos años han aparecido alternativas más sostenibles para dietas en acuicultura, como la proteína de organismos unicelulares (microalgas, bacterias y levaduras). Sin embargo, estas fuentes proteicas siguen presentando, en muchos casos, limitaciones

nutricionales y económicas. La utilización de insectos en la elaboración de dietas acuícolas ha cobrado importancia especialmente desde la década de 2010, tal como se vio en la introducción en relación con el número de publicaciones que tratan este tema. Por una parte, los insectos presentan altos niveles de proteína, con un perfil de aminoácidos adecuado, aunque su composición depende de la especie a la que pertenece el insecto, y variando según factores tales como la etapa de desarrollo del insecto, el sustrato con el cual se ha alimentado, y las condiciones de cría. Por otra parte, resultan una fuente proteica sostenible, presentan una alta eficiencia de conversión del alimento frente a la ganadería tradicional, necesitan menor cantidad de agua y suelo, y pueden ser usados como "biorreactores" para convertir desechos orgánicos en una biomasa de alto valor nutricional (Guiné et al., 2021). Existe una gran diversidad de especies de insectos, pero, entre las más estudiados y utilizados en acuicultura, destacan *Hermetia illucens* (HI) y *Tenebrio molitor* (TM) (Tran et al., 2022), pertenecientes a las ordenes Diptera y Coleoptera, respectivamente. Esto podría deberse a la posibilidad de que estas especies se críen en masa en pequeñas unidades de producción, y puedan ser alimentadas con residuos o subproductos (Kenis y Koné, 2014). Además, su producción en masa crece exponencialmente; por ejemplo, la producción mundial de HI pasó de 7.000-8.000 toneladas en 2014/15 a 14.000 toneladas en 2016 (Sogari et al., 2019).

Uno de los objetivos de esta tesis se ha centrado en la búsqueda de una posible solución que alivie los problemas derivados del uso de harina de pescado como fuente proteica en acuicultura, proponiendo la harina de insecto como alternativa. Para evaluar la idoneidad de los insectos y sus efectos en distintas especies de peces con hábitos alimentarios diversos (dorada, trucha arcoíris y tenca), se han elaborado dietas con distintos niveles de sustitución de harina de pescado por harina de insecto (HI y TM). Para ello se han evaluado diversos factores que afectan al crecimiento, al metabolismo aminoacídico, a la calidad nutritiva del filete de las tres especies mencionadas, y a la comunidad microbiana intestinal de la dorada.

Los resultados mostraron que con un 15 y 30 % de sustitución de harina de pescado por harina de insecto, la dorada fue la única especie que mostró una disminución significativa de su crecimiento cuando se alimentó con la dieta H-30, mientras que la trucha arcoíris y la tenca no se vieron afectadas tras la inclusión de harina de insecto. Esta tendencia se confirmó al aumentar los niveles de sustitución (al 50 %) de harina de pescado por harina de HI en la dieta de dorada. Las dietas compuestas por TM (al 50 %) también afectaron

al crecimiento de la dorada, aunque esta disminución no fue tan acusada como en el caso de HI. Una posible explicación a este menor crecimiento de la dorada respecto a las otras dos especies reside en la baja digestibilidad proteica aparente que estos ejemplares presentaron para las dietas que contenían HI. Tal hecho puede ser debido al mayor contenido de quitina en la cutícula de HI respecto a la de TM, y a su diferente composición, ya que, en el caso del primer insecto, se asocian al polímero altos contenidos de $CaCO_3$, que puede resultar en una menor bioaccesibilidad de la proteína unida a la cutícula (Do et al., 2021). Si, además, se tienen en cuenta los menores niveles de aminoácidos esenciales presentes en la harina de HI, se puede encontrar una posible explicación a esta diferencia en el crecimiento de dorada al aumentar los niveles de harina de HI. No obstante, la ingesta de bajos niveles de quitina puede generar efectos beneficiosos (Ringø et al., 2012; Kamilya y Khan, 2020), aunque mayores concentraciones de este polisacárido pueden limitar la absorción de proteína y lípidos (Tanaka et al., 1997). Por otra parte, la presencia de insectos en la dieta natural de trucha y tenca, ocasionada por sus hábitos alimentarios, puede favorecer una mayor predisposición digestiva en ellas para hidrolizar la quitina, de manera que la afectación de la digestibilidad proteica sea mínima. Esto, sumado a las modificaciones en las principales enzimas encargadas del catabolismo aminoacídico, permiten indagar aún más sobre el origen de la diferencia existente en el crecimiento de las distintas especies alimentadas con harina de insecto.

En general, la respuesta que hemos observado en esta tesis en el crecimiento de las distintas especies tras la inclusión de harina de insecto es variable, y parece responder principalmente a los hábitos alimentarios de los peces y a la especie de insecto incluida. En su revisión, Tran et al. (2022) observaron que la harina de HI presentaba un peor rendimiento en cuanto a crecimiento respecto a la harina de TM en dietas de acuicultura. Los resultados presentados muestran que niveles de sustitución de hasta el 30 % de HI y TM no afectan negativamente al crecimiento de tenca y trucha. En el caso de la dorada, además de las razones apuntadas anteriormente, también se ha señalado que una posible explicación al menor crecimiento observado tras altos niveles de sustitución es la sobreestimación de la proteína presente en los insectos (Hua, 2021). La presencia de nitrógeno no proteico puede llevar a una sobreestimación de la proteína en las dietas, siendo la quitina una de las fuentes más relevantes de nitrógeno no proteico en los insectos (Janssen et al., 2017). Por tanto, el menor nivel de proteína dietaría en las dietas

compuestas por insectos explicaría la disminución del crecimiento de la dorada con la dieta HI (30 %). Por un lado, al ser la dorada una especie carnívora, su necesidad proteica es superior a la tenca, y por otro lado la edad de las doradas fue inferior a la de las truchas arcoíris, por lo que su necesidad proteica aumento respecto a estas. Respecto a niveles superiores de sustitución (50 %), se aprecia un aumento de los efectos negativos en el crecimiento de la dorada, especialmente para las dietas compuestas por HI. En este experimento también se introdujo una dieta con HI (30 %) para establecer un control de referencia. La diferencia existente en el crecimiento de los peces con la dieta HI (30 %) entre ambos experimentos, aunque los piensos tenían la misma composición, parecía indicar que la causa podría provenir del tratamiento realizado a la harina de HI en el segundo experimento. El tratamiento de los insectos desde su cría y sacrificio, hasta su procesado *post mortem* influye en su composición, digestibilidad y palatabilidad (El Hajj et al., 2022). En este caso particular, el procesado de las harinas de HI puede haber sido distinto en alguno de los procesos de cría, sacrificio o secado, lo cual explicaría estas diferencias de crecimiento entre ambas dietas con un mismo nivel de sustitución por HI en distintos experimentos.

Al evaluar la calidad nutritiva de los piensos con harina de insecto, hemos de hacer un especial énfasis en la utilización de los aminoácidos dietarios y su utilización por parte de las distintas especies. La proteína se encuentra compuesta por aminoácidos, los que a su vez son una de las principales fuentes energéticas en los peces. Estos pueden destinar los aminoácidos dietarios a la obtención de energía a través del catabolismo aminoacídico, el cual se ve favorecido por la capacidad de los peces para eliminar el amonio resultante directamente a través de las branquias. Aunque el catabolismo aminoacídico es inevitable, su modificación puede revelar detalles sobre la utilización proteica de la dieta, así como la calidad de la fuente. La actividad detectada en el presente trabajo para alanina transaminasa (ALT), aspartato transaminasa (AST) y glutamato deshidrogenasa (GDH), principales enzimas del catabolismo aminoacídico, reveló la distinta utilización de los aminoácidos dietarios según la especie y dieta. En este sentido, se observó la respuesta enzimática obtenida para la dieta control en las tres especies; la tenca presentó una alta actividad para las dos transaminasas, posiblemente como consecuencia del carácter omnívoro de la especie y un exceso de proteína en la dieta (Lupiánez et al., 1989; Benzer et al., 2009). Por su parte, la trucha presentó una mayor afinidad por el sustrato asociado a la enzima ALT, lo que puede ser debido a que esta especie presenta una pobre regulación

del metabolismo hepático, debido a una baja síntesis de glucosa endógena (Panserat et al., 2000; Panserat et al., 2001; Marandel et al., 2015), por lo que utiliza el piruvato resultante de la reacción catalizada por ALT para la síntesis de glucosa a través de la gluconeogénesis (Cowey et al, 1977; Moon y Foster, 1995). La dorada mostró niveles altos en la V_{max} de AST en comparación a la trucha, aun siendo ambas especies carnívoras. Esto puede indicar distintas necesidades energéticas, ya que la alta actividad de AST está relacionada con una mayor producción de oxalacetato, el cual se destina al ciclo de los ácidos tricarboxilicos.

La inclusión de harina de insecto trajo consigo cambios en la actividad de estas enzimas. En trucha, la actividad de las dos transaminasas y de GDH aumentó tras la inclusión de este sustrato en la dieta, indicando una mayor utilización de los aminoácidos dietarios para fines energéticos. Esta situación es compatible para una dieta con niveles altos de proteína o un desbalance entre aminoácidos esenciales y no esenciales (Sánchez-Muros et al., 1998; Fournier et al., 2003; Gómez-Requeni et al., 2003; Peres y Oliva-Teles, 2006). En dorada, la actividad de ALT se vio aumentada al introducir harina de HI o TM en la dieta, lo que podría deberse a una mayor necesidad de piruvato obtenido en la desaminación de la alanina por ALT, para posteriormente ser usado en la gluconeogénesis (Fynn-Aikins et al., 1995; Ballantyne et al., 2001). Tanto ALT como AST dan lugar a productos gluconeogénicos, aunque la ALT es sustancialmente más importante (Fynn-Aikins et al., 1995). La sustitución del 50 % de harina de pescado por HI y TM no afectó a la V_{max} de ALT, AST y GDH en dorada respecto a la dieta control. Esto también fue observado por otros autores (Chemello et al., 2020; Mastoraki et al., 2020; Melenchón et al., 2021; Mastoraki et al., 2022). En tenca, por el contrario, aumentó la V_{max} de las tres enzimas para una sustitución del 30 % por HI, aunque solo fue significativo el aumento en GDH. Es posible que la menor concentración de aminoácidos esenciales en la harina de HI en comparación con la de TM pudiera provocar este aumento en las enzimas del catabolismo aminoacídico, debido a un mayor desbalance entre aminoácidos esenciales y no esenciales (Fournier et al., 2003; Gómez-Requeni et al., 2003), lo cual se ve magnificado a mayores niveles de sustitución.

Se ha visto que el catabolismo aminoacídico responde en primer lugar al nivel trófico de las especies, y en segundo lugar a las distintas necesidades y carencias del metabolismo de cada especie. En el momento en el que se inició este trabajo, no existían muchos estudios sobre la implicación de la harina de insecto en el metabolismo aminoacídico de

peces. Posteriormente han ido apareciendo nuevos trabajos que analizan la actividad hepática de ALT, AST y GDH (Guerreiro et al., 2020; Chemello et al., 2020; Mastoraki et al., 2020; Mastoraki et al., 2022; Melenchon et al., 2022), aunque en la mayoría de las ocasiones se limitan a evaluar los cambios en la V_{max}, sin analizar los distintos cambios conformacionales y de afinidad por el sustrato de las enzimas. El aumento de la actividad de las transaminasas (ALT y AST) está positivamente relacionado con el crecimiento y utilización de la proteína dietaría, mientras que su disminución puede significar un posible daño hepático (Lin y Luo, 2010; Kumar et al., 2017).

Está claro que el aumento en la actividad de estás enzimas se puede producir por un desbalance en el perfil aminoacídico de la proteína, un aumento de la proteína dietaría o una disminución en el nivel de lípidos dietarios. El desajuste existente entre el perfil de aminoácidos de la harina de pescado con el obtenido para HI o TM puede desencadenar un aumento del catabolismo de aminoácidos. Una posible solución a este problema podría ser la combinación de harinas de insecto con un perfil aminoacídico complementario; ajustando las proporciones de distintas harinas de insecto se puede lograr un perfil de aminoácidos similar al de la harina de pescado. Por otro lado, hay que poner un mayor énfasis en evaluar la fracción de proteína digerible y no digerible en los insectos. La cutícula del exoesqueleto de los insectos está formada por quitina, proteína, lípidos y, en algunos casos, compuestos inorgánicos. La proteína presente en la cutícula puede presentar una menor digestibilidad, al ser menos bioaccesible. Si se produce esta menor digestibilidad, la proporción de aminoácidos disponibles puede ser distinta a la esperada. Esto puede causar desbalances en los aminoácidos disponibles en el pool, y por tanto modificar la utilización de los aminoácidos dietarios.

Mas allá del crecimiento y utilización proteica de los peces alimentados con la harina de insecto, otro factor relevante a evaluar es la calidad lipídica de los filetes de las distintas especies y su modificación tras la ingesta de harina de insecto. Uno de los atractivos del pescado como producto son los altos niveles de n-3 LCPUFA, principalmente ácido eicosapentaenoico y docosahexaenoico (EPA y DHA), presentes en el filete destinado al consumo. Tanto HI como TM carecen de estos ácidos grasos, por lo que la inclusión de las harinas de estos insectos reducirá potencialmente la cantidad aportada a los peces. Los resultados obtenidos en el presente trabajo muestran que la inclusión de harina de insecto provocó una disminución en los niveles de EPA en el filete de dorada y trucha arcoíris. En el caso de la dorada, esta reducción fue más notable para las dietas con un mayor nivel

de sustitución, mientras que la trucha arcoíris presentó una disminución sistemática para todas las dietas con harina de insecto, siendo notablemente más bajos las concentraciones de EPA observadas para las dietas con TM. Los niveles de DHA se vieron afectados únicamente para la trucha arcoíris, repitiéndose el patrón de disminución más acusada para las dietas con TM. Así, en T-30, el nivel de DHA detectado fue la mitad del nivel observado para el filete de la dieta control. La tenca, por el contrario, no vio afectados los niveles de EPA y DHA tras la inclusión de la harina de insecto, lo cual establece diferencias con respecto a la respuesta ofrecida por la trucha, aun siendo ambos peces de agua dulce. Esta diferencia entre la tenca y la trucha en los niveles de EPA y DHA puede deberse a una menor actividad y expresión de las enzimas encargadas del metabolismo de ácidos grasos en peces de niveles tróficos superiores (Garrido et al., 2019; Xie et al., 2020). Cabe destacar que, independientemente de la especie de pez y de la dieta experimental, la proporción de DHA en los músculos fue mayor que en las dietas, lo que indica que los peces retienen selectivamente este ácido graso esencial (Panini et al., 2017).

Con relación a la composición lipídica, existen distintos parámetros que hacen referencia a la calidad del perfil de ácidos grasos presente en el filete. Uno de los más relevantes en productos acuáticos es la relación *n*-3/*n*-6 PUFA (Iaconisi et al., 2018). Mientras que los *n*-3 PUFA promueven efectos antiinflamatorios, la mayoría de los *n*-6 PUFA contribuyen a la inflamación (Rodríguez-Cruz et al., 2005). La disminución de esta relación en el filete de los peces es consecuencia de una baja relación *n*-3/*n*-6 PUFA en ambos insectos utilizados en esta tesis (Nogales-Mérida et al., 2019). Como es de esperar, la disminución de EPA y DHA en el filete de los peces alimentados con dietas con harina de insecto, especialmente en trucha, provoca una disminución de este parámetro y, por lo tanto, de la calidad lipídica. No obstante, aunque el "valor saludable" de los perfiles lipídicos de los peces que consumieron insectos empeoró con relación con los peces control, seguían presentando un nivel beneficioso de la relación *n*-3/*n*-6 PUFA. Si la concentración óptima para la salud humana oscila entre 1 y 4 (Simopoulos, 2002), todos los filetes de los peces que consumieron insectos superaron el nivel mínimo de 1,0. De cualquier manera, niveles mayores de inclusión o periodos muy largos de administración de una dieta podría conllevar la aparición de procesos patológicos en el pez. Para paliar este problema, por una parte, se propone el desengrasado como una alternativa eficiente a la hora de eliminar la fracción lipídica compuesta por ácidos grasos con valor comercial y completar el pienso con fuentes lipídicas ricas en LCPUFA. Por otra parte, en general el perfil lipídico de los

animales está influenciado por la dieta, por lo que la modificación del perfil de ácidos grasos de los propios insectos puede aumentar el valor comercial y nutritivo de la fracción lipídica de los insectos. El aprovechamiento de residuos orgánicos procedentes de peces como sustrato de cría de insectos puede mejorar el perfil de ácidos grasos del insecto, aumentado su contenido en HUFAS. De hecho, se ha observado que los insectos alimentados con sustratos ricos en PUFA n-3 modifican su perfil de ácidos grasos, aumentando los niveles de estos compuestos (St-Hilaire et al., 2007; Barroso et al., 2017; Spranghers et al., 2017; Barroso et al., 2019). Partiendo de resultados previos, se planteó la mejora nutritiva de larvas de HI y TM, usando como sustrato dos especies de pescado procedente de descartes (*Sardinella aurita* y *Pagellus bogaraveo*). De esta manera, además de enriquecer las larvas, se buscaba valorar esta opción como un posible método de reutilizar los descartes de pescado, que según el Art. 15, Reglamento (CE) No 1380/2013 deberán ser desembarcados en puerto.

Tanto la alacha (*S. aurita*) como el besugo (*P. bogaraveo*) son dos fuentes ricas en *n*-3 LCPUFA, especialmente EPA y DHA. Según lo observado, parece claro que los hábitos alimentarios de las dos especies de insectos condicionaron la ingesta de las diferentes dietas, y por tanto sus niveles de proteína y grasa. La disminución en los niveles de grasa de las larvas de TM alimentadas con las dietas compuestas únicamente por pescado refleja una baja ingesta por parte de las larvas. Además, la baja concentración de fibra en estas dietas puede producir una disminución en su correcto crecimiento, debido a que los sustratos de TM suelen ser principalmente harinas y cereales (Li et al., 2016b). En cambio, las larvas de HI aumentaron sus niveles de lípidos al ingerir dietas compuestas íntegramente por pescado, debido al carácter detritívoro de estas larvas. También aumentó la proteína para ambas larvas al aumentar los niveles de pescado en las dietas, lo que en el caso de TM fue resultado de una disminución en los niveles de grasa corporales.

El planteamiento central de este experimento se fundó en la mejora del perfil de ácidos grasos de las larvas de HI y TM, especialmente la acumulación de EPA y DHA. Claramente, la disminución de grasa corporal en TM alimentado con pescado resultó en una disminución en la cantidad de EPA y DHA acumulados. No obstante, los lípidos resultantes presentaron una mejor relación de PUFA *n*-3/*n*-6. En otro trabajo posterior realizado por nuestro grupo, en el que se evaluaron distintos métodos de pretratamiento del pescado para mejorar la ingesta por parte de TM, se encontró que el pescado fresco triturado aumentaba el crecimiento y los niveles de EPA y DHA respecto al resto de

pretratamientos (Romero-Lorente et al., 2022). Las larvas de HI mostraron un buen rendimiento a la hora de acumular EPA y DHA, resultados que confirman los estudios realizados por Barroso et al. (2017) y Barroso et al. (2019). Los datos muestran mayor cantidad en materia seca de EPA y DHA en estas larvas, principalmente debido a una mayor acumulación de lípidos por parte del insecto, ya que su cría se realizó en un estadio cercano a la pupación (Arrese et al., 2010). Los niveles máximos de EPA y DHA se alcanzaron en las larvas de HI alimentada con una dieta compuesta únicamente por besugo. Como era de esperar, el aumento de los PUFA n-3 en HI provocó un aumento drástico de la relación *n*-3/*n*-6 PUFA. La baja acumulación de *n*-3 LCPUFA podría ser consecuencia de una baja eficiencia a la hora de retener estos ácidos grasos debido a que no se encuentran en sus hábitos dietarios (Colombo et al., 2019). Por otro lado, los niveles inferiores de DHA acumulados en comparación a EPA, pueden deberse a que los insectos metabolizan este ácido graso para obtener energía. Weers y Gulati (1997) observaron un comportamiento similar para *Daphnia galeata* alimentada con una dieta rica en DHA.

El uso de insectos como "bioacumuladores" de proteínas y lípidos de alta calidad constituye un método sostenible para aprovechar el pescado de descarte cuando la producción de harina de pescado no es posible, debido a la falta de infraestructura o al bajo rendimiento económico. El Reglamento (CE) 142/2011 no permite la alimentación de insectos con proteínas animales no procesadas, como el pescado de descarte, (artículo 3.6 del Reglamento (CE) 1069/2009). Se espera que nuevos estudios y ensayos de seguridad alimentaria permitan establecer un nuevo marco legislativo para dar cabida a estrategias como las expuestas en este trabajo.

Para determinar el efecto que tiene la harina obtenida mediante alimentación de insectos con descartes de pescado frente a la harina de insecto obtenida alimentada con pienso comercial se diseñó un experimento con dietas para dorada compuestas por harina de insectos (TM y HI) con altos niveles de sustitución (50 %). Por un lado, se elaboró una dieta con insectos alimentados con pienso comercial (H-50 y T-50) y otra con el mismo nivel de sustitución, pero con HI mejorada, es decir, alimentada con pescado de descarte (H-50M). Esta mejora se realizó de acuerdo con los datos descritos en el ensayo anterior y los estudios realizados por Barroso et al. (2017) y Barroso et al. (2019). Desafortunadamente, los resultados obtenidos no fueron los esperados en comparación con la harina de HI no mejorada (H-50). Tanto los parámetros de crecimiento como los de utilización dietaría no se acercaron a los obtenidos para la dieta control o la dieta con

alta sustitución de harina de pescado por TM (T-50). El perfil de ácidos grasos del músculo de la dorada (**Anexo I-Tabla A**), aunque no fue finalmente incluido en el trabajo publicado, no mostró una mejora significativa en los niveles de EPA y DHA respecto a una dieta con el mismo de nivel de sustitución por HI no mejorada.

Tal y como se comentó al discutir el crecimiento observado al principio de esta sección, los resultados negativos obtenidos para todas las dietas compuestas por HI, tanto mejorada como no, puede deberse al tratamiento aplicado en la elaboración de la harina. En este nuevo experimento se volvió a preparar una dieta compuesta por HI al 30 % de sustitución para ejercer como dieta de referencia a la hora de comparar dos experimentos. Los parámetros de crecimiento y utilización dietaría empeoraron respecto a la misma dieta en el primer experimento, indicando que los peores resultados obtenidos se deben a un tratamiento distinto de las larvas de HI a la hora de elaborar la harina. Para la harina HI mejorada, las larvas se alimentaron con pescado, aunque nuestro equipo no tomo parte en la cría de estos insectos, por lo que el proceso de cría o el sustrato usado no puede ser confirmado.

Sin embargo, y a pesar de no lograr el objetivo buscado, está harina presentó una mayor digestibilidad aparente de la proteína en comparación con las dietas compuestas por HI a cualquier nivel de sustitución. Esto puede estar relacionado con la población microbiana encontrada para la dieta H-50M, que presentó un alto grado de semejanza con la comunidad microbiana presente en el intestino de las doradas alimentadas con la dieta control. La principal diferencia fue la presencia de especies con capacidad quitinolitica, como *Bacillus subtilis* (Wang et al., 2018), lo cual podría permitir la hidrolisis de la quitina presente en la cutícula, aumentando así la bioaccesibilidad de la proteína presente en esta.

La importancia de la comunidad microbiana intestinal es grande, ya que estos microorganismos participan en procesos fisiológicos, nutricionales y de desarrollo del hospedador (Ye et al., 2014). Estas poblaciones pueden llegar a producir enzimas digestivas distintas a las endógenas, que facilitan la hidrolisis y absorción de nutrientes (Ray et al., 2012), además de producir compuestos esenciales según las necesidades particulares de cada especie (Mountfort et al., 2002; Tsuchiya et al., 2008). El microbioma intestinal de los peces se ve modificado por varios factores, tales como la dieta y el hábitat, aunque existe un núcleo de especies invariablemente asociado a una misma especie, independientemente de cuáles sean las condiciones de desarrollo. Conociendo las

diversas funciones desempeñadas por la microbiota intestinal, cabe esperar que las diferencias en el crecimiento de las doradas, alimentadas con dietas compuestas por harinas de insecto, puedan estar estrechamente relacionadas con la población microbiana. Bajo esta hipótesis se analizó el microbioma intestinal de doradas alimentadas con dos dietas compuestas por HI a dos niveles de sustitución (H-30 y H-50), una dieta compuesta por HI mejorada (H-50M), una dieta compuesta por TM (T-50) y una dieta control. Los resultados mostraron que la variación del microbioma respondía principalmente al nivel de sustitución de la harina de pescado por harina de insecto. Mientras que la diversidad del microbioma presentó mayores diferencias para la dieta H-30 respecto al control, la dieta H-50M mostró una mayor similitud a la dieta control en cuanto a la variedad de las comunidades microbianas. Se observó que especies de *Vibrio* y *Bacillus* eran predominantes en el intestino de la dorada. En ese sentido, la alta presencia de *Vibrio harveyi* en los tratamientos C y H-50M, se vio rebajada para los tratamientos H-30, H-50 y T-50, mostrando una mayor presencia de otras especies del mismo género. Dado que este grupo cuenta con especies con efectos beneficiosos (Carnevali et al., 2016), su incremento en detrimento de la abundancia relativa de *V. harveyi*, especie con capacidad patogénica, puede tener un componente positivo. Aunque los tratamientos C y T-50 no compartieron la misma diversidad del microbioma, ambas dietas se agruparon conjuntamente en lo que respecta a los parámetros de crecimiento, así como varios de los procesos metabólicos analizados.

Respecto a los grupos microbianos menos predominantes, se encontraron diferencias entre los tratamientos para la presencia de varias bacterias con efectos positivos. En las dietas compuestas por harina de insecto se detectó la presencia de *Sulfitobacter pontiacus* y miembros de la familia Alterominadaceae. Estas especies pueden degradar materia orgánica en medios acuáticos y actuar como probiótico, respectivamente. Mientras que estas especies no aparecieron en el tratamiento con la dieta control, otras especies como *Streptococcus dysgalactiae*, *Peptostreptococcus russellii*, y *Weisella confusa* siguieron un patrón opuesto, no detectándose prácticamente en el intestino de las doradas alimentadas con dietas compuestas por harina de insecto. Estas bacterias, que se han relacionado con procesos digestivos y antibacterianos, además de actuar como promotores del crecimiento (Pieterse y Todorov, 2010; Califano et al., 2017; Tenea y Lara, 2019; Fowler et al., 2021; Rastekenari et al., 2021), mostraron una relación positiva frente a varios parámetros de

crecimiento, así como de utilización de la dieta, apuntando la posibilidad de que su presencia en el microbioma intestinal pueda ejercer un papel clave.

En definitiva, hemos comprobado que la sustitución hasta un 30 % de harina de HI o TM no produce cambios drásticos en el crecimiento o en la utilización de aminoácidos en la dorada, trucha arcoíris o tenca. De estas tres especies, la dorada fue la única con un crecimiento inferior para la dieta compuesta por HI (30 %); esta disminución se vio agravada cuando la sustitución alcanzó el 50 %. La inclusión de los lípidos de los insectos, carentes en EPA y DHA, reduce los niveles de PUFA *n*-3 en la dieta y consecuentemente en el filete de los peces. Se ha comentado previamente como el tratamiento de la harina de insecto influye en su digestibilidad, palatabilidad y niveles de lípidos. Los estudios sobre el tratamiento preliminar a la harina de insecto van en aumento (El Hajj et al., 2022). El fraccionamiento mecánico de los insectos puede resultar, por un lado, en una fracción compuesta principalmente por exoesqueleto y otra fracción rica en proteínas y lípidos. El exoesqueleto contiene quitina, la cual puede desencadenar procesos alérgicos, reducir la absorción de nutrientes y afectar negativamente al crecimiento en peces. Esta fracción rica en quitina puede someterse a diversos tratamientos químicos para extraer la proteína presente en la cutícula del exoesqueleto y obtener quitina con un alto nivel de pureza. La quitina presenta un amplio espectro de aplicaciones, entre las cuales se encuentra su uso como matriz de encapsulación a través de su derivado desacetilado, quitosano. Considerando esta capacidad, para finalizar y bajo el mismo marco de sostenibilidad establecido a la hora de realizar la tesis, se intentó abordar la problemática existente al respecto del uso de antibióticos en acuicultura y a su vez aplicar quitosano en la búsqueda de alternativas.

En la introducción se habló de como el uso de antibióticos en la industria acuícola supone un gran problema por diversos factores. Entre los principales problemas asociados a está practica se encuentran la generación de resistencia a los antibióticos por parte de las bacterias, efectos sobre la diversidad del ecosistema, y efectos en la microbiota del hábitat (Song et al., 2016; Okocha et al., 2018; Okeke et al., 2022). El número de antibióticos y los casos en los que se pueden aplicar dentro de la industria acuícola se ha visto restringido en varios países (Lulijwa et al., 2019; Pepi y Focardi, 2021), aunque en regiones como África y América del Sur se estima que su uso aumente para el 2030 (Schar et al., 2020). Esta situación abre la posibilidad e invita a la búsqueda de alternativas más sostenibles para el tratamiento de infecciones en acuicultura. A este respecto, los

polifenoles del té verde son compuestos con un gran rango de actividades biológicas beneficiosas, entre las cuales se incluye actividad antibacteriana (Yanagawa et al., 2003; Taguri et al., 2006; Thawonsuwan et al., 2010; Wang et al., 2017; Ji et al., 2018; Zhang et al., 2020; Álvarez-Martínez et al., 2021; Qian et al., 2021; Zhang et al., 2021; Li et al., 2022). Los polifenoles del té verde se componen principalmente por catequina y dentro de estás, la epigalocatequina galato (EGCG) es la mayoritaria y, a su vez, la que mayor actividad biológica ha demostrado tener (Singh et al., 2011; Kim et al., 2014; Yan et al., 2020). Sin embargo, estas moléculas son lábiles y se degradan con facilidad en medio fisiológico, reduciendo así su absorción y vida media (Dang et al., 2015; Rambaran, 2020; Di Santo et al., 2021).

Como ya se ha referido en numerosas ocasiones, los insectos y su aplicación en la acuicultura son el tema central de esta tesis. A lo largo de este documento se han discutido de forma extensa los distintos efectos relacionados con la sustitución de harina de pescado por harina de insectos. En muchas ocasiones, el peor desempeño de algún parámetro se ha visto relacionado con la ingesta de la quitina presente en el exoesqueleto de los insectos. La quitina, tal y como se ha comentado, es un factor que en ciertas cantidades afecta al desarrollo y estado saludable de los peces (Lock et al., 2018; Tran et al., 2022). El quitosano es el producto de la desacetilación de la quitina, y su mayor diferencia con esta reside en la solubilidad en medio ácido y, al igual que la quitina, presenta una gran variedad de aplicaciones (Khoushab y Yamabhai, 2010; Morin-Crini et al., 2019; Kozma et al., 2022). Entre estas aplicaciones, podemos destacar su papel en la nanotecnología, donde puede actuar como matriz de encapsulación, así como estabilizante de nanopartículas (Han et al., 2016; Khan et al., 2019; Cheba, 2020; Zhou et al., 2021). En este último apartado de la tesis se ha evaluado la encapsulación de EGCG o extracto de té verde en nanopartículas de zeína estabilizadas con alginato, para posteriormente estudiar los efectos que presentan estás nanopartículas tras la incorporación de una capa de quitosano. El resultado fue nanopartículas con un tamaño creciente tras la sucesiva adición de capas a las nanopartículas de zeína, aunque la adición de dichas capas confirió una mayor estabilidad a la dispersión. Del mismo modo, la adición de las capas de alginato y quitosano aumentó la encapsulación de EGCG y el extracto de té verde (GTE), debido a que estos polisacáridos presentan grupos funcionales que incrementan la interacción entre las moléculas encapsuladas y la matriz de encapsulación (Yuan et al., 2022). Aunque finalmente no fue incluida en el trabajo, se obtuvo información respecto

a la estabilidad de las diferentes nanopartículas obtenidas a diferentes concentraciones de NaCl (**Anexo I-Tabla B**), detectándose que las nanopartículas recubiertas por quitosano mostraron una mayor estabilidad a altas concentraciones salinas lo que resulta especialmente interesante para su uso en acuicultura.

Los ensayos de actividad antioxidante pusieron de manifiesto una menor actividad antioxidante en todos los formatos de nanopartículas respecto a las sustancias libres. Esto puede ser debido a que las moléculas se encuentran formando enlaces de hidrogeno con la matriz de encapsulación (Li et al., 2009), lo cual puede reducir la capacidad antioxidante de los polifenoles (Gulcin, 2020). Por otro lado, la capacidad antioxidante de EGCG fue superior al extracto de té verde, lo que propició también una mayor actividad antioxidante de las nanopartículas con EGCG. Esta diferencia en la actividad antioxidante se debe a que EGCG es el polifenol con mayor actividad antioxidante del té verde, y aunque en este caso formaba el 50 % del extracto de té verde, la concentración de EGCG era inferior a la que presentaba el compuesto puro. La pérdida parcial de la capacidad antioxidante de las sustancias encapsuladas se ve compensada por la mayor estabilidad que le confiere la nanocápsula (Khan et al., 2019; Pauluk et al., 2019; Liang et al., 2021).

La actividad antimicrobiana de las nanopartículas fue probada frente a cinco distintas bacterias patogénicas de peces, cuatro Gram-negativas y una Gram-positiva. La presencia de quitosano en las nanopartículas fue el factor más determinante en la inhibición del crecimiento de las bacterias. Sin embargo, la presencia de EGCG o extracto de té verde no trajo consigo una mejora de la actividad antibacteriana para las cinco especies bacterianas, en conjunto. Las nanopartículas con quitosano pueden presentar actividad antibacteriana por si solas, tal y como se ha descrito en otros estudios (Friedman et al., 2013; Al-Gethami y Al-Qasmi, 2021). Si individualizamos la acción de las nanopartículas para cada especie bacteriana, se observa que la especie con menor sensibilidad a las nanopartículas para todos los formatos de compuestos antimicrobianos probados fue *Vibrio anguillarum*, mientras que *Photobacterium damselae* fue la más afectada por la acción de los distintos tratamientos. Por otro lado, la acción de las nanopartículas con EGCG o GTE fue similar siempre que la nanocapsula tuviera la misma composición. Tanto EGCG como GTE en su forma libre no mostraron niveles de inhibición comparables a sus homólogos encapsulados. En términos económicos, el primero de estos

resultados es positivo, ya que el uso de un compuesto puro siempre es más costoso que el uso de un extracto sin purificar.

Todo lo anteriormente expuesto pone de relieve la complejidad de establecer conclusiones genéricas a la hora de incluir las harinas de insectos en dietas de acuicultura. Como se ha visto, el efecto en la digestión y asimilación de una misma especie de insecto difiere entre la especie de pez estudiado, y dentro de la misma especie de pez, la respuesta es muy variable dependiendo del insecto y nivel de sustitución incluido. Esto muestra que, aunque la investigación sobre el uso y aprovechamiento de los insectos en acuicultura está creciendo exponencialmente, existen enormes lagunas de conocimiento, y será necesario hacer un esfuerzo mayor sobre el valor nutritivo de las diversas especies de insectos y su aprovechamiento por sus consumidores potenciales.

Las mayores limitaciones para emplear los insectos en acuicultura son: el alto contenido graso de las larvas, la escasa proporción de LCPUFA *n*-3 en su perfil de ácidos grasos, o el efecto negativo de la quitina del exoesqueleto, entre otros. Es necesario valorar en profundidad el efecto de tratamientos como la eliminación/reducción del exoesqueleto, el desengrasado, tratamientos térmicos (como el escaldado o el extrusionado) en el valor nutritivo, digestibilidad y palatabilidad de los insectos.

Otro aspecto fundamental, que hasta ahora no se ha estudiado suficientemente es la relación entre la microbiota del insecto y de los peces, y los efectos colaterales tanto en la salud, asimilación y productividad de los peces de acuicultura.

Y, por último, creemos haber mostrado el papel crucial que ejerce el quitosano, proveniente de la quitina presente en el exoesqueleto de insectos, en la síntesis de nanomateriales. Estos nanomateriales pueden ser usados en la encapsulación de sustancias bioactivas, con efectos positivos en organismos acuáticos.

Creemos que el futuro de los insectos como alimento alternativo es ya indiscutible, no obstante, para su uso generalizado debe resolverse algunos limitantes todavía. Además de los cambios en la legislación, ya apuntados, habría que añadir:

- El precio de mercado debe disminuir. Como la demanda es actualmente limitada, los precios deben adaptarse al tamaño de los pedidos (Gasco et al., 2020), y, por lo tanto, las harinas de insectos todavía no son competitivas, con relación con otras fuentes de proteínas.

- Estandarización de las harinas de insectos. El contenido de nutrientes varía mucho, no sólo entre las especies de insectos, sino incluso dentro de la misma especie. Las características de los nutrientes dependen de la etapa de vida, el entorno, la dieta, los métodos de procesamiento o sacrificio, etc. Esto limita su uso en la industria de los piensos, ya que es necesario contar con una disponibilidad de materia prima de calidad homogénea y estable.
- Romper las barreras culturales. Estas barreras, presentes principalmente en los países más desarrollados económicamente, podrían superarse con una correcta información sobre la sostenibilidad de su producción y su composición nutritiva. Y parece que los consumidores aceptan comer animales alimentados con piensos que incluyan insectos (Verbeke et al., 2015). Y en concreto, Mancuso et al. (2016) encontraron que casi el 90 % de los consumidores estaba dispuesto a comer pescado alimentado con insectos.

CONCLUSIONES

1. El perfil nutricional de los insectos resulta adecuado como alternativa parcial a la harina de pescado. Aunque presenta limitaciones, tales como alto contenido en grasa bruta y bajos niveles de *n*-3 LCPUFA. **Capítulos I y II.**
2. Es posible incrementar moderadamente los niveles *n*-3 LCPUFA en insectos mediante el uso de sustratos ricos en estos ácidos grasos, especialmente para *H. illucens*. **Capítulo III.**
3. Bajo nuestras condiciones experimentales, una sustitución moderada de harina de pescado por harina de insecto (máximo 30 %) no implica efectos destacados sobre parámetros de crecimiento, mientras que niveles superiores sí lo hacen. **Capítulos I y IV.**
4. De los insectos estudiados, *T. molitor* mostró mejores cualidades como sustituto de la harina de pescado, ya que los peces alimentados con la harina de este insecto presentaron mejores índices nutritivos y de crecimiento. **Capítulos I y IV.**
5. La inclusión de harina de insecto no afecta al metabolismo proteico de forma especial, siendo los factores de mayor peso el desbalance aminoacídico de la harina de insecto y los hábitos alimentarios de las especies de peces estudiadas. **Capítulo I.**
6. La inclusión de harina de insecto en la dieta para peces no afecta los niveles de *n*-3 LCPUFA en el filete de tenca, mientras que provoca una disminución de estos especialmente en el de trucha arcoíris. A pesar de esta disminución, el filete mantiene un "valor saludable", al mantener un nivel beneficioso en la relación *n*-3/*n*-6 PUFA. **Capítulo II.**
7. La inclusión de harina de insecto modifica el microbioma intestinal de *S. aurata*, siendo más influyente el nivel de inclusión que la especie de insecto usada. La alimentación del insecto con sustratos ricos *n*-3 LCPUFA disminuye la variación de la microbiota respecto a los peces control. **Capítulo IV.**
8. La obtención de nanomateriales que incluyen quitosano permite nuevas estrategias para el tratamiento de enfermedades infecciosas en acuicultura. **Capítulo V.**

Conclusión final:

En definitiva, con niveles moderados de sustitución, la harina de insectos han demostrado ser un ingrediente adecuado en acuicultura. No obstante, esta depende tanto de la especie de insecto como del pez acuicultivado. Para incluir niveles superiores de estas harinas son necesarias futuras investigaciones sobre el sistema de cría de los insectos, así como del tratamiento y procesado de sus harinas. Por otra parte, el efecto antimicrobiano de

quitosano resulta prometedor en la aplicación de tratamientos y prevención de enfermedades, y sus aplicaciones deben ser estudiadas con mayor profundidad.

CONCLUSIONS

1. Although it has limitations, such as high crude fat content and low levels of *n*-3 LCPUFA. **Chapters I and II.**
2. It is possible to moderately increase n-3 LCPUFA levels in insects by using substrates rich in these fatty acids, especially for *H. illucens*. **Chapter III.**
3. Under our experimental conditions, a moderate substitution of fishmeal by insect meal (maximum 30 %) has no effect on growth parameters, whereas higher levels do. **Chapters I and IV.**
4. Of the insects studied, *T. molitor* showed better qualities as a substitute for fishmeal, as fish fed with fishmeal from this insect showed better nutritional and growth indices. **Chapters I and IV.**
5. The inclusion of insect meal does not affect protein metabolism in any particular way, being the most important factors, the amino acid imbalance of the insect meal and the feeding habits of the fish species studied. **Chapter I.**
6. The inclusion of insect meal in the fish diet does not affect the levels of *n*-3 LCPUFA in tench fillet, while it causes a decrease in these levels especially in rainbow trout fillet. Despite this decrease, the fillet maintains a "healthy value" by maintaining a beneficial level in the *n*-3/*n*-6 PUFA ratio. **Chapter II.**
7. Inclusion of insect meal modifies the gut microbiome of *S. aurata*, with the level of inclusion being more influential than the species of insect used. Feeding the insect with *n*-3 LCPUFA-rich substrates decreases the variation of the microbiota compared to control fish. **Chapter IV.**
8. The development of nanomaterials including chitosan enables new strategies for the treatment of infectious diseases in aquaculture. **Chapter V.**

Final conclusion:

In summary, with moderate levels of substitution, insect meal has proven to be a suitable ingredient in aquaculture. However, this depends on both the insect species and the fish aquacultured. In order to include higher levels of these meals, further research on the insect rearing system, treatment and processing of the insect meals is needed. On the other hand, the antimicrobial effect of chitosan shows promise in the application of disease treatment and prevention, and its applications need to be studied further.

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ANEXO I: TABLAS SUPLEMENTARIAS

Tabla A. % de EPA y DHA respecto al total de ácidos grasos en músculo de *Sparus aurata* alimentada con dos dietas con harina de HI y una dieta control.

	EPA			DHA		
C	4,12	±	0,07	17,90	±	0,29[a]
H-50	3,41	±	0,09	12,22	±	0,51[b]
H-50M	3,22	±	0,36	12,58	±	0,42[b]

Media±error estándar. Diferentes letras significan diferencias significativas ($p<0,05$).

Tabla B. Tamaño y estabilidad de nanopartículas de zeína recubiertas con alginato y/o quitosano en diferentes concentraciones de NaCl.

Diámetro hidrodinámico (nm), índice de polidispersidad (PDI) y potencial ζ (mV, ZP) de nanopartículas de zeína recubiertas por alginato.

NaCl	Diámetro hidrodinámico			PDI			ZP		
50 mM	442.3	±	25.0	0.267	±	0.014	-30.3	±	0.72
150 mM	La dispersión se agregó y precipitó.								

Diámetro hidrodinámico (nm), índice de polidispersidad (PDI) y potencial ζ (mV, ZP) de nanopartículas de zeína recubiertas por alginato y quitosano.

	Diámetro hidrodinámico			PDI			ZP		
50 mM	250,5	±	1,28	0,195	±	0,007	33,36	±	0,69[a]
150 mM	251,3	±	1,39	0,170	±	0,007	24,10	±	0,28[b]
350 mM	261,1	±	2,60	0,166	±	0,011	16,40	±	0,49[c]
500 mM	272,9	±	27,12	0,171	±	0,010	17,26	±	1,03[c]

Las nanopartículas de zeína no se muestran en la tabla debido a que la dispersión se agregó con una concentración de 50 mM NaCl. Media±error estándar. Diferentes letras significan diferencias significativas ($p<0,05$).

ANEXO II: OTRAS PUBLICACIONES CIENTÍFICAS

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