Optimización de piensos para trucha arco iris (Oncorhynchus mykiss) mediante la valorización de harinas de insecto

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Tesis Doctoral – PhD Thesis

Optimización de piensos para trucha arco iris (*Oncorhynchus mykiss*) mediante la valorización de harinas de insecto

Optimization of feeds for rainbow trout (*Oncorhynchus mykiss*) through the valorisation of insect meals

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Abbreviation key

AA: amino acid
ADC_{prot}: apparent digestibility coefficient of the protein
CAT: catalase
DHA: docosahexaenoic acid
DPA: docosapentaenoic acid
EPA: eicosapentaenoic acid
EU/EU 27: European Union
FA: fatty acid
FAO: Food and Agriculture Organization (of the United Nations)
FBP_{ase}: fructose-1,6-bisphosphatase
FIFO: fish-in, fish-out
FM: fishmeal
G6PDH: glucose-6-phosphate dehydrogenase
GDH: glutamate dehydrogenase
GIT: gastrointestinal tract
GOT: glutamate oxaloacetate transaminase
GPT: glutamate pyruvate transaminase
GPx: glutathione peroxidase
GR: glutathione reductase
HI: black soldier fly (*Hermetia illucens*)
IM: insect meal
LC: long chain
MDA: malondialdehyde
MUFA: monounsaturated fatty acid
n-3: omega-3
n-6: omega-6
OTU: operational taxonomic unit
PER: protein efficiency ratio
PK: pyruvate kinase
PPV: productive protein value
PUFA: polyunsaturated fatty acid
SDG: sustainable development goals
SFA: saturated fatty acid
SOD: superoxide dismutase
TM: yellow mealworm (*Tenebrio molitor*)
USD: United States Dollar

Resumen

La acuicultura es una industria alimentaria con un increíble potencial debido a que los peces disponen de eficientes índices productivos, una buena calidad de proteína y elevados niveles de ácidos grasos esenciales para el ser humano. Sin embargo, este mismo potencial tiene sus costes, tales como una elevada demanda fisiológica de proteína. Dicha demanda ha sido tradicionalmente satisfecha utilizando harina de pescado, un ingrediente que procede mayoritariamente de la pesca extractiva y que se considera insostenible. Por esta razón, tanto la investigación como la industria llevan ya bastantes años buscando fuentes de proteína alternativas, tales como las harinas de insecto. Los insectos tienen también buenos índices productivos y calidad de proteína, así como un buen potencial a nivel de sostenibilidad, pero a cambio tienen sus contrapartidas. Las desventajas mejor conocidas de los insectos como ingredientes para peces son probablemente dos: sus elevados costes, y su baja concentración de ácidos grasos poliinsaturados de cadena larga omega-3, que se transmiten por medio de la dieta. Como para cualquier otro ingrediente novedoso, las harinas de insecto requieren de investigación para conocer mejor sus límites de inclusión y sus repercusiones en la fisiología de peces.

Esta tesis amplía lo que se sabe sobre esos puntos por medio de tres estudios en diferentes etapas de desarrollo (juvenil, crecimiento, y finalización) de trucha arcoíris (*Oncorhynchus mykiss*), reemplazando parcialmente la harina de pescado de la dieta con dos harinas de insecto enteras: una procedente de la mosca soldado negra (*Hermetia illucens*) y otra del gusano de la harina (*Tenebrio molitor*). En el tercer estudio, centrado en el gusano de la harina como ingrediente, se busca compensar la bajada de omega-3 por medio de dos estrategias: el empleo de una versión desengrasada de esta harina de insecto, y el enriquecimiento de la dieta con un aceite experimental rico en omega-3. Como principales resultados de la tesis, el gusano de la harina muestra un rendimiento ligeramente superior en la especie de destino, relacionado con su mayor digestibilidad, y mayor longitud de microvellosidad intestinal. Las dietas basadas en insectos han mostrado mayor actividad digestiva a nivel intestinal, y no se han observado cambios en la función hepática. Aunque las causas no se conocen, también tienden a potenciar la respuesta antioxidante y el sistema inmunológico de los peces. La inclusión de gusano de la harina favoreció el crecimiento de bacterias probióticas en la microbiota intestinal, lo cual estuvo muy influido por la composición grasa del insecto. Respecto a la calidad del filete de pescado, el cambio más remarcable es el descenso de omega-3 en los mismos, que se puede solventar con diferentes estrategias. También se ha observado un efecto ahorro de omega-3 que ha derivado en una mayor acumulación grasa en hígado y músculo. Los cambios observados en filete no han modificado el perfil organoléptico del mismo. Por último, la etapa de desarrollo de los peces resultó también relevante a la hora de la evaluación de distintas respuestas fisiológicas.

Los resultados de esta tesis remarcan el potencial de las harinas de insecto como fuente alternativa de proteína para su uso en dietas para trucha arco iris. Como principales perspectivas de futuro, se propone expandir aquellos puntos que aún quedan como incógnita, tales como descubrir los mecanismos bioquímicos que provocan el estímulo de la respuesta antioxidante y la inmunológica, o aquellos otros en los que no se ha ahondado lo suficiente, como la naturaleza del efecto ahorro de omega-3 en los filetes.

Summary

Aquaculture is a food industry with incredible potential due to the high productive indices of fish, their good protein quality, and high levels of essential fatty acids for human consumption. However, this potential comes with costs, such as a high physiological demand for protein. Traditionally, this demand has been met by using fishmeal, an ingredient mostly derived from extractive fishing practices and considered unsustainable. For this reason, both research and industry have been searching for alternative protein sources for many years, such as insect meal. Insects also have good productive indices and protein quality, as well as a good sustainability potential, but they also have their downsides. The best-known disadvantages of insects as fish feed ingredients are probably their high costs and their low concentration of long chain omega-3 polyunsaturated fatty acids, which are transmitted through the diet. Like any other novel ingredient, insect meals require research to better understand their inclusion limits and their impact on fish physiology.

This thesis expands what is known about these points with three studies at different developmental stages (juvenile, growth, and finishing) of rainbow trout (*Oncorhynchus mykiss*), replacing partially the fishmeal in the diet with two whole insect meals: one derived from the black soldier fly (*Hermetia illucens*) and the other from the yellow mealworm (*Tenebrio molitor*). In the third study, focused on yellow mealworm as an ingredient, two strategies are employed to compensate the reduction in omega-3: the use of a defatted version of this insect meal and the supplementation of the diet with an experimental omega-3-rich oil. As the main results of the thesis, yellow mealworm shows a slightly higher performance in the target species, related to its higher digestibility and longer intestinal villi height. Insect-based diets have shown higher digestive activity at the intestinal level, and no changes in liver function have been observed. Although the causes are unknown, they also tend to enhance the antioxidant response and immune system of the fish. The inclusion of yellow mealworm favors the growth of probiotic bacteria in the intestinal microbiota, which is greatly influenced by the composition of insect fat. Regarding fish fillet quality, the most remarkable change is the decrease in omega-3 content, which can be addressed with different strategies. It was also observed an omega-3 sparing effect that resulted in greater fat accumulation in the liver and muscle. The changes observed in the fillet have not modified its organoleptic profile. Finally, the developmental stage of the fish was also relevant in the evaluation of different physiological responses.

The results of this thesis emphasize the potential of insect meals as an alternative protein source for their use in rainbow trout diets. As the main future perspectives, it is proposed to expand on those points that remain unknown, such as discovering the biochemical mechanisms that cause the enhancement of the antioxidant and immune responses or those others that have not been sufficiently investigated, such as the nature of the omega-3 sparing effect in fillets.

CHAPTER 1 – General introduction

GENERAL INTRODUCTION

1. State of aquaculture: Fish consumption and aquaculture

Aquaculture is the process through which different products can be cultivated in an aquatic environment. Nowadays, it is considered one of the fastest growing industries when it comes to talking about the production of food because it has grown about a yearly 7.5 % since 1970. It is expected to keep on growing to be able to produce approximately the 53 % of fish for human consumption in 2030 [1]. Taking into account the good growth ratios that can be found in aquaculture species and the comparatively better efficiency of these animals at turning feeds into edible fractions when compared with terrestrial farmed animals [2], this is a good sign towards the future of humanity as a sustainable society.

At the time of writing this introduction, global population is numbered as 8.01 billion [3], with an expected growth up to 8.5 billion for the year 2030, 9.7 billion for 2050 and 10.4 billion for 2100 [4]. In September 2015, the United Nations launched its 2030 Agenda for Sustainable Development, where 17 Sustainable Development Goals (SDG) were described [**Figure 1**] [5]. Due to these reasons, it is no surprise that in 2018 the FAO mentioned aquaculture as one of the best assets to face the increasing global demand of protein [6]. Previously, a document was published involving the relevance of aquaculture towards the fulfilment of these SDG. Numbers two, "Zero hunger", eight, "Decent work and economic growth", twelve, "Responsible consumption and production" and fourteen, "Life below water" were highlighted [7].

According to the latest FAO reports [1,8], the total aquatic production (aquaculture plus extractive fishing) reached a peak of 214 million t in 2020, including finfish, aquatic algae, crustaceans, molluscs and other invertebrates. This number represents a 0.3 % increase from the previous record of 2018 (213.4 million t), which can be considered a minor improvement. However, it would be worthwhile to mention that this limited growth was motivated by a 6.2 % decrease in the production of extractive fishing practices (mostly due to COVID-19 pandemic), while the global production of aquaculture was able to compensate it. A 57 % of aquatic production comes from aquaculture (122.6 million t), representing, in economic terms, a total value of 281.5 billion USD at first sale. World aquaculture production was distributed as: 46.8 % fish (57.4 million t; 49.1 million t from inland aquaculture and 8.3 million t from marine and coastal aquaculture), 28.6 % algae (35.1 million t), 14.5 % molluscs (17.7 million t), 9.2 % crustaceans (11.2 million t) and 0.9 % of other aquatic animals (1.1 million t) such as amphibians or sea cucumbers; 55.6 % of species were sea water based (marine or coastal aquaculture), while 44.4 % of species were fresh water based (inland aquaculture) [**Figure 2**] [1].

Referring to use, and excluding algae and other aquatic plants (35.1 million t), the global fishery production reached 177.8 million t in 2020, 88.5 % for human consumption (157.4 million t), representing an apparent consumption of 20.2 kg *per capita*. The remaining 11.5 % was mostly used (81 % of it; 16 million t) to be reduced to fishmeal (FM) and fish oil [1]. From the total for human consumption (157.4 million t), 87.5 million t come from aquaculture, representing a 55.6 % [**Figure 3**].

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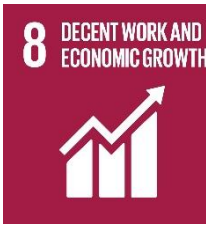
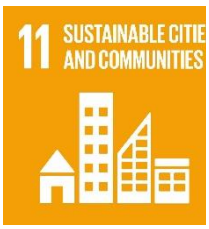
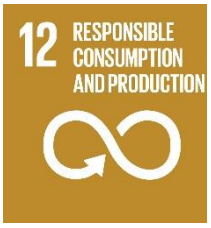
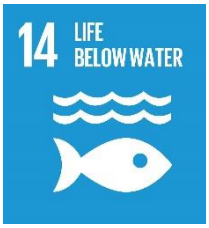
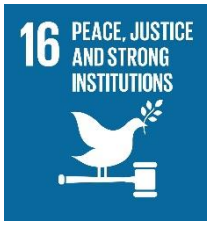
	++		+++		++
	+		+		+++
	++		+++		++
	+		++		

Figure 1. Relevance of aquaculture in the fulfilment of the FAO Sustainable Development Goals (SDG). Relevance score: + potentially relevant, ++ relevant, +++ highly relevant. Data and pictures taken from FAO [7].

Productions were very different depending on the analysed region. In 2020, and from the perspective of continents, Asia was the main producer of aquaculture representing the 91.6 % of total production (112.3 million t). After Asia, the Americas would come in a second place with a 3.6 % of global production (4.4 million t). Europe would be third with 2.69 % of global production (3.3 million t), followed by Africa (1.9 %, 2.4 million t) and Oceania (0.2 %, 0.2 million t) [1].

From a perspective of countries, the production of Asia was extensively led by China, which accumulated 57.5 % of the total world production, 56.7 % for aquatic animals (49.6 million t), and 59.5 % for algae (20.8 million t). Indonesia had the second highest aquaculture production with 12.1 % of world production (14.8 million t), but almost two thirds of its production belonged to algae (9.6 million t). Its production of aquatic animals (5.2 million t) was behind that of India (8.6 million t, 7 % of total world), which almost represented the totality of its aquaculture production. Viet Nam (3.8 % of world total, 4.6 million t), Bangladesh (2.1 %, 2.6 million t), Egypt (1.3 %, 1.6 million t) and Chile (1.2 %, 1.5 million t) would follow as the next countries in global aquaculture production, all of them being represented by their aquatic animal production in almost a 100 % [1].

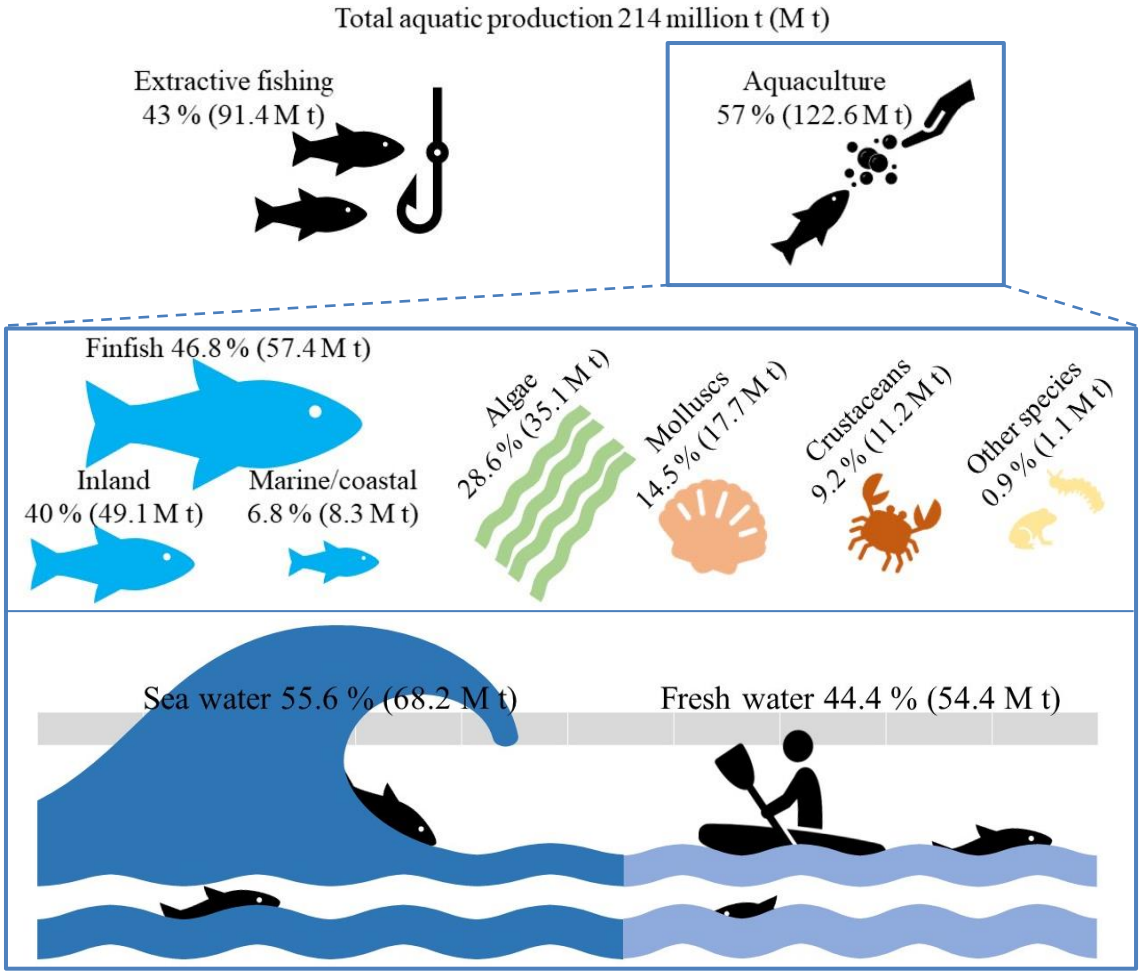


Figure 2. World global aquatic and aquaculture production referring to origin in 2020. Data taken from State of World Fisheries and Aquaculture 2022 [1].

Putting the focus on Europe, the first producer was Norway with a production of 1.5 million t of aquatic animals (1.2 % of total, and 45.3 % of Europe), surpassing the production of the European Union-27 (1.1 million t, 33.2 % of European production) and the rest of the European countries (0.7 million t, 21.5 % of Europe) [1]. Spain would stand at the twenty second position in the world with 276,562 t [9], third position in Europe closely behind Turkey.

Excluding algae, the top produced species in world aquaculture of 2020 were the Pacific white shrimp (*Penaeus vannamei*, 5.81 million t), the grass carp (*Ctenopharyngodon idellus*, 5.79

million t), the Pacific oyster (*Crassostrea* spp., 5.45 million t) and the silver carp (*Hypophthalmichthys molitrix*, 4.89 million t). In economic terms, *P. vannamei* would make this species reach a first place, meaning a total of 33.7 billion USD (12 % of the total sale value of world aquaculture production, 281.5 billion of United States dollar), red swamp crayfish (*Procambarus clarkii*) being at a relatively far second place with 21 billion USD. The main focus of this thesis, rainbow trout (*Oncorhynchus mykiss*), appears in the ninth place with 953,299 t and a first sale value of 4.3 billion USD [9].

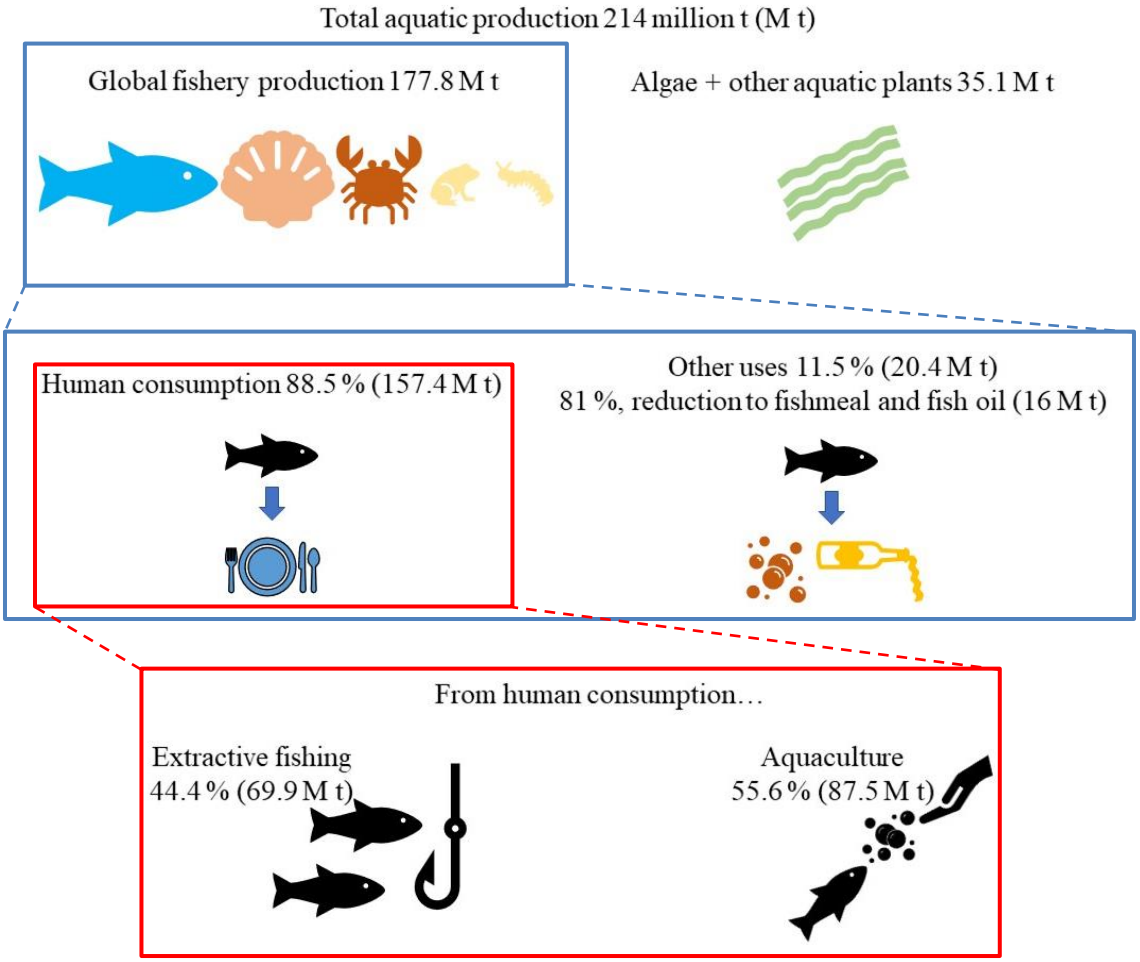


Figure 3. World global aquatic production referring to use in 2020. Data taken from State of World Fisheries and Aquaculture 2022 [1].

After the exit of the United Kingdom from the European Union (EU 27), the aquaculture data drops a total of 1.09 million t [**Figure 4**], valued as 4,222 million € in EU 27. The main production are finfish (552,622 t) and molluscs (537,571 t); marine finfish represents 42 % (229,953 t) of the total finfish, against a 39 % of diadromous finfish (216,476 t) and a 19 % of freshwater finfish (106,193 t). The main species produced are the mussels (*Mytilus spp.*), followed by rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (*Sparus aurata*). Spain appears as the biggest EU 27 producer with a 25 % of the total volume (276,562 t), but due to the relatively low value of mussel, it was the second country on the list of production value (657.3 million €), France being the first one with 828.4 million €. In reference to finfish production (with a total of 552,622 t from EU 27 production; 50.7 % of total), Greece appears as the first country with 112.2

t and a value of 624.5 million €, Spain being the second producer with 66.5 t and 510.1 million € [9].

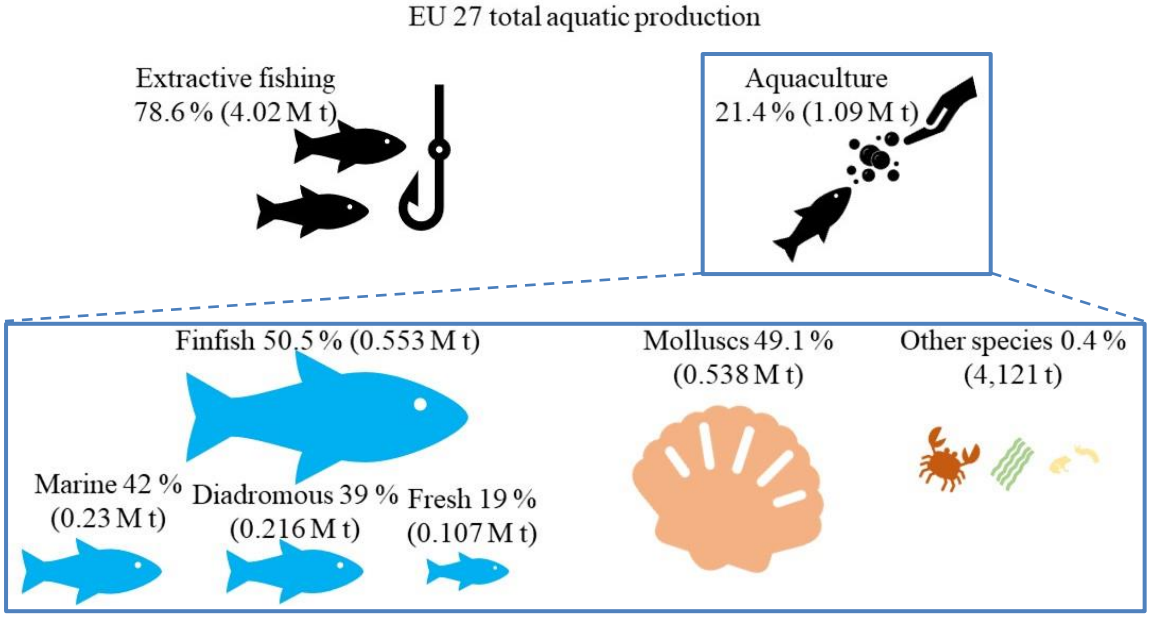


Figure 4. Total aquatic production in European Union (EU 27; 2020). M t: million ton. Data taken from FishStatJ [9].

According to the Spanish Agriculture, Fishing and Food Ministry [10], a total of 272,882 t and 563.3 million € were cultured and sold, respectively, in Spain, in 2020, representing the 26 % of total Spanish aquatic production [**Figure 5**]. The Spanish aquaculture is dominated by molluscs (206,749 t), especially mussel, with a 74.8 % of the volume production against a 24.1 % of finfish production (66,545 t). With respect to farmed finfish, the marine species constitute a 75.6 % (50,282 t) led by 22,765 t of seabass (*Dicentrarchus labrax*), 8,913 t of red tuna (*Thunnus thynnus*), 6,963 t of turbot (*Scophthalmus maxima*) and 6,458 t of seabream (*Sparus aurata*). Diadromous species meant a 24.4 % of farmed finfish, led by rainbow trout (*Oncorhynchus mykiss*) with 15,806 t. Pure freshwater species meant only a production of 7.12 t [9].

2. The rainbow trout and its production

The rainbow trout (*Oncorhynchus mykiss*; Walbaum 1792) is a carnivorous teleost fish from the family Salmonidae. As mentioned by Stankovic [11], the rainbow trout "*is a diverse assemblage of various subspecies and forms that exhibit a great range of variation in all characteristics used for classification. This plastic species inhabits both freshwater and marine habitats of western North America and Northeast Asia*". Due to this variation, the external characteristics of rainbow trout, and especially its colour, can change with habitat, size and sexual condition. This document will focus on the adult appearance of the most typical freshwater variation of the fish that can be found in fisheries. This species has a long, laterally compressed body, and the following characters can be used to differentiate it from other trout species: a fleshy adipose fin, which appears between the dorsal and the (forked) caudal fin; an anal fin with 6-9.5 (usually 8.5) branched rays; a mid-lateral row with between 115 and 130 small cycloid scales; 16-17 gill rakers and between 60 to 66 vertebrae; the colour varies from blue to olive green over

a pink-iridescent band that runs above the lateral line, and a silver band below it; the dorsal side, flanks, head and fins are covered with small black spots. The overall aspect of the fish varies from an intense dark coloration to a bright-silver one. Adult freshwater stream rainbow trout average between 0.5 and 2.5 kg, while cultured rainbow trout are usually harvested at 0.25-0.7 kg in freshwater (depending on the region) or 4-5 kg in marine cages. It is an anadromous salmonid able to adapt to seawater. The largest individuals of the species measured over 1.2 m in length and 25 kg in weight, but it is described a more common maximum length of 60 cm and maximum weight of 10 kg.

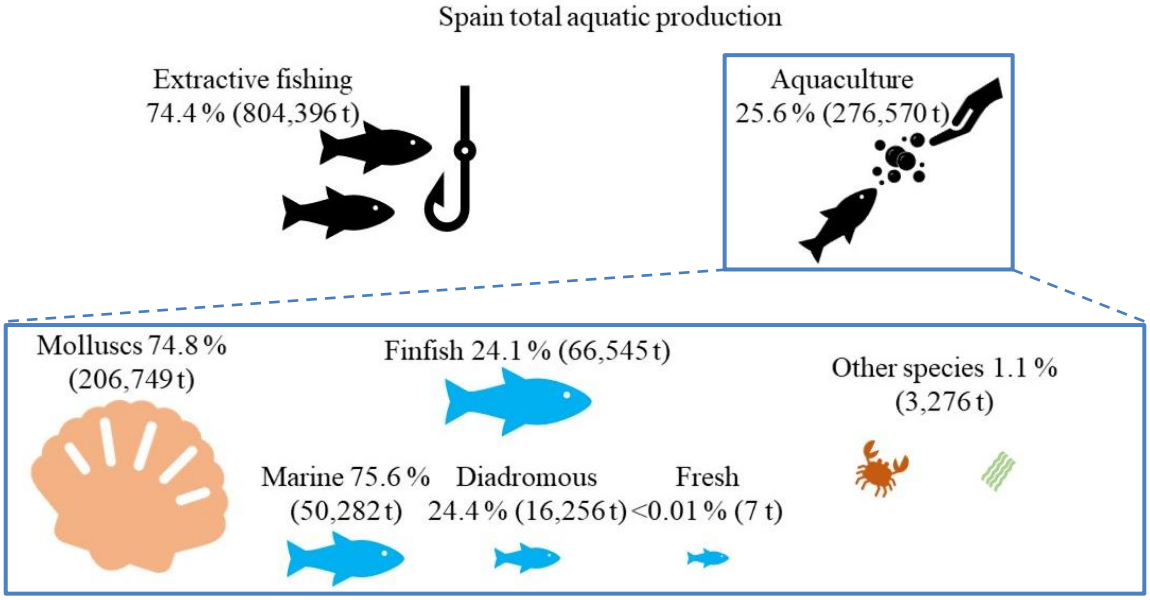


Figure 5. Total aquatic production in Spain (2020). Data taken from FishStatJ [9].

Reproductively, it develops as a synchronous differentiated gonochoristic teleost with an annual reproductive cycle and a marked sexual dimorphism. Females can produce up to 2,000 eggs (3-7 mm of diameter) per kg of body weight [12-14]. In rearing conditions, a simultaneous presence of fish males and females can generate size dispersion during fattening, as well as cannibalism and social hierarchies [15]. Rainbow trout males show precocious sexual maturation and, consequently, exhibit lower growth rates at early stages in contrast to females [16]. It is for these reasons that the production of all-female rainbow trout lots are preferred for aquaculture. They are usually produced through one of the following methods: a) masculinization of rainbow trout, where females are treated with a dose of 17-alpha-methyl testosterone (typically in feeding), producing a sexual reversion that leads to individuals with female genotype and male phenotype, and the sperm of these individuals is then used to fertilise normal female ova to create an offspring of 100 % XX females [17]; b) production of sterile triploids, where post-fertilised fish ova are given a shock treatment (temperature, pressure or radiation) to produce the retention of the second polar body of the oocytes, creating a generation of XXX or XXY sterile females [18].

Concerning the productive data of this species in aquaculture, a total of 862,335 t were cultured globally in 2018, 903,225 t in 2019 and 959,690 t in 2020. The biggest proportion of cultured rainbow trout is produced in freshwater (76 %, 733,999 t), but another big part of its production cycles is finished in salt water after a preadaptation (smoltification) process, especially in Chile and Norway. The EU 27 contributed 168,812 t in 2018, 183,816 t in 2019 and 183,506 t

in 2020 [9]. In Spain, the production of rainbow trout [**Table 1**] was 16,160 t in 2018, grew up to 16,939 t in 2019, and decreased to 13,620 t in 2020 and 15,357 t in 2021. In 2021, the main Spanish producers of rainbow trout were the Autonomous Regions of Castilla y León (3,834 t), La Rioja (1,781 t), Galicia (1,750 t), Cataluña (1,650 t), Aragón (1,609 t), Andalucía (1,550 t), Asturias (1,100 t), Navarra (1,036 t) and Castilla la Mancha (842 t) [19].

Table 1. Spanish production of rainbow trout per year (t). Data taken from APROMAR report, 2022 [19].

Spanish production of rainbow trout (t)	2018	2019	2020	2021
Castilla y León	4,975	5,888	3,617	3,834
La Rioja	1,728	1,912	1,728	1,781
Galicia	2,689	1,840	1,363	1,750
Cataluña	1,630	1,921	1,652	1,650
Aragón	1,191	1,098	637	1,609
Andalucía	1,380	1,448	1,587	1,550
Asturias	704	922	1,160	1,100
Navarra	791	856	685	1,036
Castilla la Mancha	773	797	991	842
Cantabria	184	168	129	150
País Vasco	115	89	66	55
Comunidad Valenciana	0	0	6	0
Total	**16,160**	**16,939**	**13,620**	**15,357**

3. Protein in fish nutrition

Due to the fast growth of the aquaculture sector, this industry has its own problems when talking about its relationship with the natural environment. In general, fish are known for having bigger protein requirements within their digestive and metabolic demands than homeotherm animals. Most cultured fish species are carnivorous, which have even bigger protein demands than herbivorous/omnivorous fish [20]. Traditionally, this protein demand of fish has been satisfied with one particular group of ingredients: FM, which mostly come from whole wild-caught fish (73 %) or as a by-product (27 %) of extractive fishing practices [1]. FM proved to be optimal for a long time because its composition is great to fulfill the protein requirements of fish, but as years and decades kept going, this meant a complex and serious sustainability concern. Aware of this problem, both scientific community and the feed industry have invested resources and efforts to decrease the inclusion of FM in grower diets as a main protein source in aquafeeds [1]. In this vein, nowadays it is not strange to find the "Fish-In, Fish-Out" ratio concept in technical literature, usually referred to by its acronym, "FIFO ratio". This term is a practical concept to get a quick approach of the number of fish (usually, as kg of FM) needed to produce 1 kg of cultured fish. It is known that, indeed, this FIFO ratio has been drastically reduced during the last decades, moving from higher than 2 in 1992, to less than 0.5

in our days [**Figure 6**] [22]. As stated previously, global population is expected to keep growing, meaning a need for more protein production for human consumption purposes. This need invites the creation of protein factories, aquaculture fisheries being a great option. The consequential rapid development of aquaculture leads to an inevitable and big increase in protein demand for millions of cultured fish, and consequently the demand of FM. Thus, the relative FIFO numbers look promising, but more work is needed to keep lowering these cyphers by means of the refinement of processes and the development of new sources of protein.

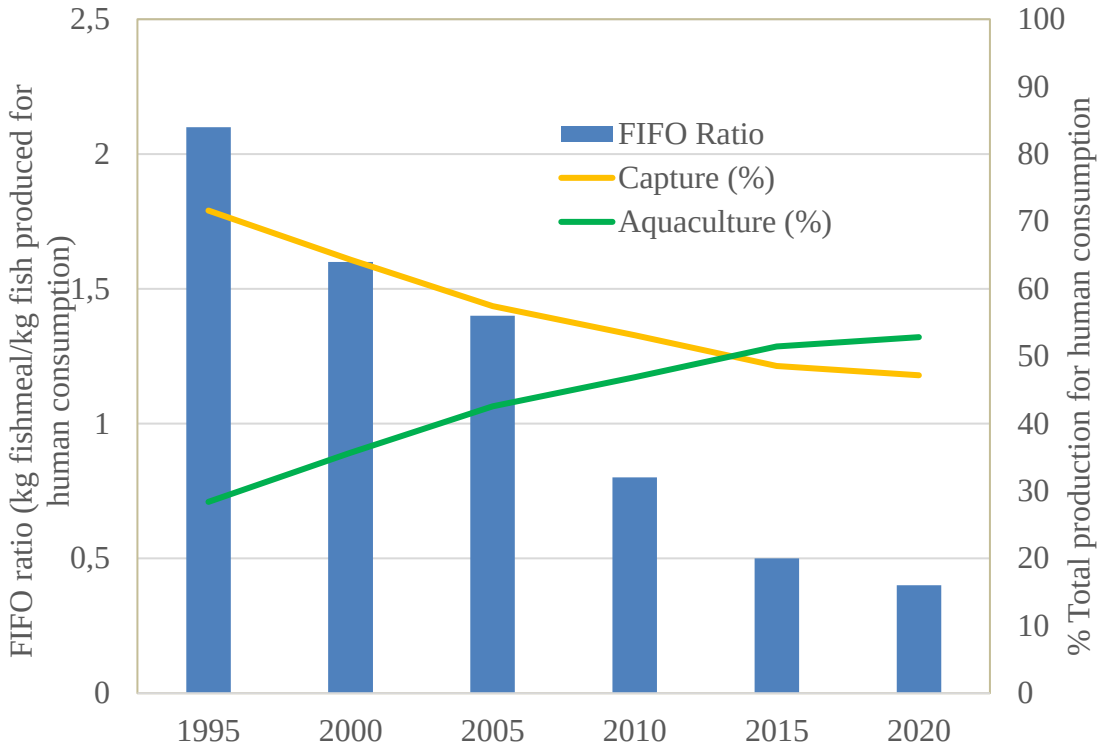


Figure 6. Aquaculture growth respect to capture fish for human consumption, and the evolution of the FIFO (Fish-In, Fish-Out) ratio (data taken from Kok *et al*., 2020 [21] and FishStatJ [9]).

4. Alternative raw materials to fishmeal as protein sources for aquaculture feeds

Although FM is still one of the most important ingredients when it comes to the supply of protein, several alternatives have been researched. The most typical alternatives to FM as protein sources have a vegetable origin, soybean meal being possibly the most widely used in animal feeds. It is known that vegetable ingredients, including soybean meal, can contain antinutritive factors, which have negative effects on the palatability of the feed (decreasing the daily feed intake), on the gastrointestinal tract (GIT), or altering the fillet quality. [22-26]. With a more positive perspective, numerous studies related to the beneficial and adverse effects of these ingredients have made it possible for its inclusion in fish feed formulations to become a reality as part of the protein fraction. However, these ingredients need a large portion of land

because they come from agriculture, and they depend on several factors that are hard to control such as availability, climatic conditions and input prices [27,28].

4.1. Other animal protein sources

Taking aside the case of insects, which will be the main focus of this document, other animal sources of protein have been researched and tested for a long time due to their rich compositions. Ingredients such as poultry and swine by-products, feather meals, meat and bone meals, or even other sea-sourced ingredients like krill meals or fish silages have already proven their efficiency during fish growth trials [29,30]. However, it is important to remember that the use of some of these resources is strictly regulated (or banned, in the case of meat and bone meals) due to the transmissible spongiform encephalopathy epidemic of 1986 [31]. This, on the other hand, should not discourage completely their use. It is known that following the current EU regulations concerning the prohibition of use of ruminant protein, as well as banning its intraspecies recycling, the risk of a new transmissible spongiform encephalopathy epidemic becomes negligible. Furthermore, the refinement of rendering technologies and the possible development of new ones could bypass the apparition of prions [32].

4.2. Seaweed

The term “seaweed” does not have a real taxonomic value, but it is a popular way of describing large marine algae. This includes brown (Phaeophyceae), red (Rhodophyceae) and green (Chlorophyceae) macroalgae [33]. As an interesting fact, it was mentioned that many seaweeds have a relatively high quality of protein, which means that their proportion of essential amino acids (AA) against the total AAs profile is quite high when compared with other protein sources such as soybean meal or even FM [34]. Some seaweeds have already proven their efficiency as low inclusion level ingredients for aquaculture feeding trials [35,36]. Since some species have shown interesting capacities such as high crop productivities per unit area [37], or the possibility of growing on by-products that would otherwise be considered as waste [38,39], they show a promising future.

4.3. Microalgae

Microalgae have also proven their efficiency in different aquaculture feeding trials [36]. Due to big differences among species, they are known to cover wide ranges in their proximate compositions. Protein content can go from as low as a described 6 % for *Spirogyra*, to as high as a 71 % for *Spirulina maxima*. Similar statements could be said for carbohydrates (from 2.9 to 64 %) and lipids (from 1.9 to 45 %), making them very interesting options as either feed formulation additives, or even main sources of macronutrients [40-43]. In addition, like seaweed, they have showed their potential to take part in an integrated system waste-nutrient to obtain a sustainable protein source for aquaculture [44]. However, the most unique features of microalgae are probably an astoundingly high proportion of essential long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA) in the composition of some species and the presence of pigments like beta-carotenes and astaxanthin [41,45-47].

4.4. Yeast

Yeasts are known to have about 40-55 % of crude protein in their composition. Similar fish growth results have been obtained in experiments where a partial replacement of FM with different yeasts was evaluated against FM-based diets [48-50]. Possibly due to the peculiar composition of yeast cell walls [51], these microorganisms have demonstrated the ability to enhance different organic fish functions when included in fish feedings, like the innate immune response of European seabass (*Dicentrarchus labrax*) [52], and the survivability, growth, and feed acceptance of gilthead seabream (*Sparus aurata*) larvae [53]. Last but not least, it would be worthwhile to mention the sustainability potential of yeast production, since it is possible to grow them on different substrates, including non-food biomass [54,55].

4.5. Other single cell protein

Stepping aside of the field of microalgae and yeast, there are other single cell organisms that are being researched as protein alternatives, bacterial protein being one of the most interesting. Some species of bacteria have really high proportions of protein that can surpass a 70-80 % of their compositions [56]. Among them, some are known as methane-oxidising species [57] that can grow on natural gas (methane) or methanol, potentially contributing to the concept of circular economy. These raw materials for feeds have already proven their efficiency in aquaculture species like rainbow trout (*Oncorhynchus mykiss*) [58] and Atlantic salmon (*Salmo salar*) [59]. Their protein digestibility has been reviewed as well, showing promising results [60].

5. Use of insect meals as protein source in aquafeeds

5.1. Basic concepts and legal framework

With close to one million described species, Insecta is the biggest class inside the Animalia kingdom [61]. These animals are known for having great levels of protein and fat, but most western countries do not have a tradition of using insects as a source of nutrients, food or feed. Instead, they are mostly perceived with disgust [62]. Recent advances in research and against social prejudices have led to a better understanding of these animals as a very interesting possibility for the creation of efficient and healthy methods to produce animal protein. The EU have officially taken some first steps to introduce insects in the food chain through the Commission Regulation (EU) 2017/893 [63]. Due to the transmissible spongiform encephalopathy outbreak of 1986, the Regulation (EC) Nº 999/2001 [64] established strict regulations on the use of most animal protein sources for the feed of farmed animals. In 2017, the cited regulation allowed using processed animal protein from seven insect species for, among other, aquaculture feeding purposes, if those insects were fed under specific conditions. These insect species were: black soldier fly (*Hermetia illucens*), common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*) and field cricket (*Gryllus assimilis*). In 2021, through the Commission Regulation (EU) 2021/1372 [65], the

reach of the 2017 regulation was broadened, making it cover more species of destination like poultry and swine cattle.

Respect to its direct use as food, the Regulation (EU) 2015/2283 [66] applied to the placing of novel foods on the markets within the Union, including whole insects or their parts. Following this regulation, entities like the European Food Safety Authority (EFSA) and private companies have been working on the authorization of the inclusion of different insects within the human food chain, such as yellow mealworm [67,68] or migratory locust (*Locusta migratoria*) [69]. Although the industrial production of insects is still at an early stage, the cited legal framework encourages its development. In this regard, the role of the International Platform of Insects for Food and Feed (IPIFF) should be highlighted, an entity which promotes the use of insects and insect-derived products for human consumption and as source of nutrients for animal feeds.

5.2. Insects for aquafeeds

When considered as an ingredient for fish feeds, and when compared to other protein sources, insects demonstrate potent advantages. Talking about their composition, the most studied insects have relatively high proportions of both protein and fat, making them look like interesting candidates for fish feed formulations. Their protein fraction is known to have a balanced composition when compared to the needs of fish, since their essential AA profile is considerably similar to that of FM [70,71]. Considering the huge amount of species within this class, which would ideally mean lots of different body compositions [72], they also show potential to satisfy the specific needs of different fish species that are being, and might be cultured worldwide. Furthermore, these animals are known for having the interesting capability of modifying substantially their own body compositions according to their diet, especially their FA profile [73,74], which makes for a strong point to support the previously mentioned. In addition, insects grow and reproduce very quickly when compared to other macroscopic species, which is crucial from an industrial production point of view. Also, because they lack many complex functions such as the internal temperature regulation of homeothermic animals, their productive ratios and ecological footprints are very efficient [75-78]. The most commonly studied insects can grow on a wide range of substrates, making them both a sustainable alternative and an interesting tool towards the concept of circular economy [79-81]. Indeed, there is even a possibility of using these animals for other purposes that do not involve the food chain, such as the production of animal biofuels from the recycling of organic waste [82,83]. Some fish species like rainbow trout are known to have insects within their natural diet in wild conditions [84,85]. In this way, it is not hard to assume that many fishes could be adapted to appropriately consume and digest insect components. Finally, insect-based ingredients could be considered as functional. It has been described that there are antimicrobial substances in their composition like lauric acid, or some antibacterial peptides like Hf-1 [86-88]. Several experiments have described a tendency to enhance the antioxidant and immunological systems of different aquaculture species, typically attributed to the chitin content of insect meal (IM) or some of its metabolites [89-91].

The two insect species that were tested in the research behind this thesis were the black soldier fly (*Hermetia illucens*), and the yellow mealworm (*Tenebrio molitor*). Before going into the details of the physiological variables that were evaluated, it would be interesting to offer a broad introduction for these species.

Black soldier fly (*Hermetia illucens*, HI) is a 2 cm long, black, wasp-like fly (Diptera order) which belongs to the Stratiomyidae family. The species is native from the tropical/subtropical zones of America, but the development of international transportation since the 1940s resulted in the spreading of this species to many regions of the world such as tropical and warmer regions between about 45ºN and 40ºS [92,93]. The larvae have a pale colour, can reach up to 27 mm in length and 6 mm in width, and weigh up to 220 mg in their last larval stage [94,95]. During this stage, they have the ability of feeding on a broad range of organic substrates even when they are on advanced levels of decay, which includes animal manure and human excreta. The larval stage can last from 2 weeks to 4 months depending on the availability of feed and the environmental conditions [92,96]. A pupal stage follows, lasting between 2 weeks and up to 5 months, depending on the environment [92]. The adults rely on the fats stored from the larval stage [97], and two days after emerging, they mate and lay their eggs next to a feed source [96]. One interesting and practical point of HI is that they act as a natural protection against those unsanitary traits that are traditionally associated with other flies. Unlike, for example, houseflies (*Musca domestica*), black soldier flies can process and dry manure very quickly, diminishing odours and reducing the harmful bacteria of these substrates. Also, the adults are not disease vectors and are not attracted to human foods or habitats [75,98].

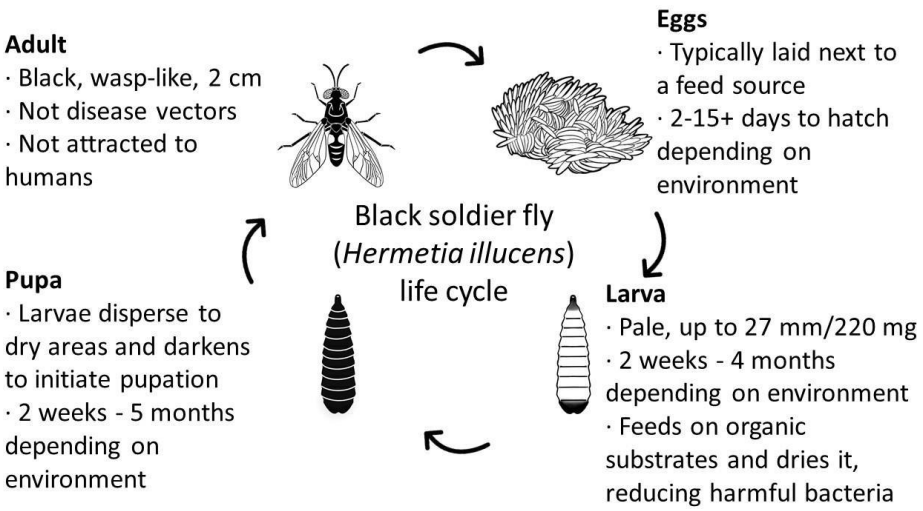


Figure 7. Life cycle of black soldier fly (*Hermetia illucens*). Source: illustrations by Daniel Avilés González.

Mealworms belongs to the Coleoptera order and the Tenebrionidae family [95]. The larvae of two different darkling beetles can be found under the common name of mealworms: the yellow mealworm (*Tenebrio molitor*, TM); Linnaeus, 1758), and the dark or mini mealworm (*Tenebrio obscurus*; Fabricius, 1792) [92]. For the purposes of this thesis, only the case of TM will be addressed. TM beetles are indigenous to Europe, but they are distributed worldwide. The name itself gives the idea that this species is a pest of grain, flour and food stores [99]. When the

larva is fully developed, it has a light yellow-brown colour, is 28–32 mm long and weighs 130–160 mg. The life cycle of TM may last from 280 to 630 days depending on environmental conditions, and begins with tiny, sticky eggs that are quickly concealed by dirt and substrate. Larvae hatch after 10–12 days (at 18–20 ºC). The larval stage typically lasts for 2-4 months (up to 18 months if environmental conditions are not optimal) and is followed by a pupal stage of 7-9 days (at 25 ºC). The adult TM lives for two to three months [92]. TM are omnivorous and can eat all kinds of plant materials (including low quality plant waste) as well as animal products such as meat and feathers, turning them into high quality protein [99]. However, the most typically used substrates include cereal bran or flour (wheat, oats, maize), and protein sources like skimmed milk powder or soybean flour. It is also recommended to provide a source of water like fresh fruits and vegetables, because even though they can survive and reproduce with almost no water, an adequate relative humidity can substantially increase their yearly fertility [92,100].

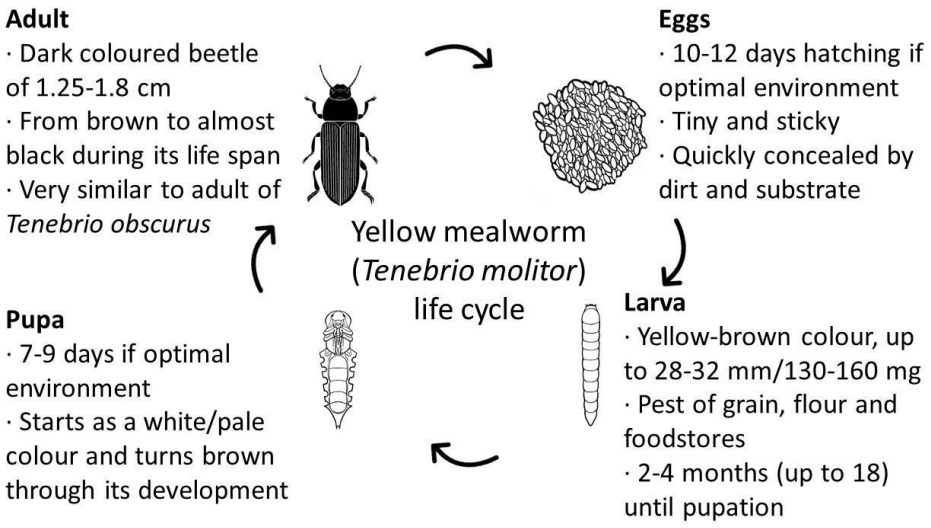


Figure 8. Life cycle of yellow mealworm (*Tenebrio molitor*). Source: illustrations by Daniel Avilés González.

5.3. Insects in aquafeed research

Due to the benefits previously mentioned, IMs as ingredients for fish have been tested for some time now. At the moment of writing these lines (January 2023), and as a quick approximation, a search on Scopus database under the terms “insect meal fish” drops a total of 409 research manuscripts, from 1941 to present. Strictly speaking, some among the first of these trials were carried out decades ago [101], but it was not until the year 1989 that the term “insect meal” was described for an experiment with rats [102]. A better aimed search discarding those articles not related to first hand experiments with insect-fed fish brings a better approximation with around 223 manuscripts, 45 of which were focused in the study of rainbow trout. If other related articles are added to the total (like book chapters, reviews and meta analyses), a continuity of these publications can be found from the year 2011 [103-105], but it is not until the years 2015-2017 that a real interest on these novel ingredients was developed [**Figure 9**].

Looking a little deeper into this information, it is easy to spot that the most typically researched insects for fish feedings are the same two species used for this thesis: HI and TM. So far, HI is the obvious winner of this competition with around 148 manuscripts published on research journals with international relevance, meaning approximately a 66 % of the total; TM would come in second place with 61 published articles, meaning around a 24 % of all insect-fed fish research manuscripts.

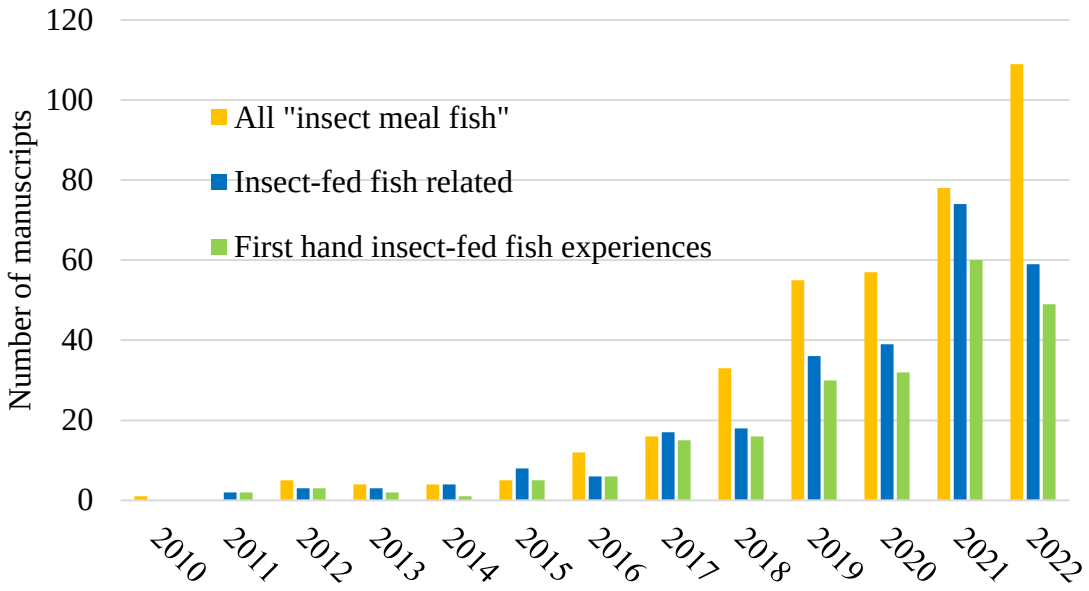


Figure 9. Number of manuscripts related to insect-fed fish (Scopus database), from 2010 to 2022.

5.4. Effect on physiological status of fish

As any novel ingredient that enters the food chain, IMs are being tested and researched to prove their adequacy from several points of view, such as their sanitary acceptability, their productivity, or even their environmental and economic sustainability. In this way, the project in which this thesis was based evaluated the repercussions of using two insects in the feeding formulations of rainbow trout for the following topics and parameters.

5.4.1. Growth performance and protein use

Growth is the most relevant parameter analysed in a majority of studies related to nutrition because improving it, most of the time, is the final and practical objective of this vast field of work. As stated previously, FM has traditionally satisfied the biggest part of the protein requirements of fish due to the excellent nutritional profile of this ingredient. This is an undoubted advantage which turns its replacement into a real challenge, because other useful and promising alternatives tend to lack some important components. Even though it depends on the species, it is known that IMs tend to be low in some AAs, with the case of TM for methionine and lysine being particularly accused on this [70,95]. However, this does not mean that IMs coming from HI and TM cannot replace FM appropriately. Numerous trials have proven that a partial replacement of FM with IMs is definitely possible without impairing fish growth [106-109]. Sometimes, this happens even when the level of FM replacement is high or complete [110-112].

As it could be expected, there are different results between these experiments. For example, it seems that carnivorous fresh water species like rainbow trout can manage from small to average proportions of IMs in their feeds without problems [109,113-115]. Sea water species, and especially percids like meagre, tend to show worse performances [116-119]. Recent meta-analyses reveal that a minor to medium inclusion of IMs in fish feedings have no negative influences on fish growth, supporting the previous statement [120-122].

The growth performance can be measured from different points of view like weight gain or food conversion ratio. However, due the fact that the main organic and edible component of a healthy animal is protein, concurrently, the efficiency in the use of the protein is also very related to growth. Values like apparent digestibility coefficient of the protein (ADC_{prot})[1], productive protein value (PPV)[2] and protein efficiency ratio (PER)[3] give a worthy approximation on the use of the protein. In other words, they reflect the path that the protein follows from the feed to the synthesis of animal tissue. As stated in a previous point, IMs are known to have a relatively high proportion of protein in their compositions [75], as well as a balanced AA profile that is partially related to that of FM [70]. However, one of their main components that brings havoc into this equation is chitin. On the one hand, it has been described that the chemical composition of chitin interferes with the most typically used methods to quantify protein. This makes it necessary to re-adjust the factors for the calculations that follow this quantification[4] [123]. Some studies have suggested that high levels of chitin tend to diminish the digestibility of fish diets and specially their protein [124-126]. However, the biggest part of the physiological repercussions of this molecule and its derivatives still remains a big incognita, and there are also experiments that described little to no disruption in protein digestibility for IMs [127-129], or that even described an improvement [130]. Overall, it can be assumed that a small proportion of chitin in the feed should not compromise protein digestibility in an important way, with due interspecific differences [131].

5.4.2. Digestive enzymes

In a similar way than protein use, digestion inside the GIT plays a key role during the first steps of nutrient assimilation. The different digestive enzymes that can be measured in the GIT can change drastically depending on several factors such as the fish species that are object of the study and/or their feeding habits [132-135], the tested ingredients/diets [118,136,137], the GIT portion that was studied [107] or the time elapsed between the last feeding period and the sampling [138,139].

As it was stated in a previous point, Insecta is the biggest class inside the Animalia kingdom, which multiplies the possible combination of factors (fish/insect species, feeding habit and GIT portion among others). Considering the previously mentioned and the big surge of research studies around insect-fed fish, it would be worthwhile to mention that there is a relatively low amount of literature on this precise topic. For example, for tilapia (*Oreochromis niloticus*) it

[1] Apparent digestibility coefficient of the protein: $ADC_{prot} = 100 - ((\text{marker in diet (g)} / \text{marker in faeces(g)}) \cdot (\%\text{ protein in faeces} / \%\text{ protein in diet}) \cdot 100)$
[2] Productive protein value: PPV = ((protein gain (g) / protein intake (g)) · 100)
[3] Protein efficiency ratio: PER = (total weight gain (g) / protein intake (g))
[4] Nitrogen-to-protein conversion factor of 4.67 for *Hermetia illucens*, and of 4.75 for *Tenebrio molitor* (Janssen *et al*., 2017) [123]

was described an increase in pepsin [140], alkaline proteases and amylase activities [141], but also a decrease in alkaline protease activity [142]. Another study highlighted an overall decrease in alkaline protease activity for African catfish (*Clarias gariepinus*) when the amount of IM in the diet was increased [143]. The case of meagre (*Argyrosomus regius*) was similar [117], while two other studies went in the opposite direction, describing an increase in alkaline proteases for juvenile grass carp (*Ctenopharyngodon idellus*) [136], or this same increase coupled with a decrease in acid proteases for gilthead seabream (*Sparus aurata*) [118]. As such, it is uneasy to draw early schemes about the repercussions of IMs on fish digestive enzymes, but looking at these results, it seems that the acid/alkaline proteases ratio tends to go down with the addition of IMs, which suggests the idea that these ingredients might be more actively digested within the intestine. The most obvious point, however, is that the complexity of variables involved in this field of work strongly encourages further research.

5.4.3. Intestine health: histology and microbiota

After digestive enzymes have fulfilled their purpose by reducing the size of the feed and simplifying its components, the absorption of these smaller particles takes place. For fish, this is a process that happens mostly in the latest part of the GIT: the intestine. While the intestine of fishes can be very different, salmonids are equipped with: a short portion with numerous, slender projections called pyloric caeca; a brief, thin portion called proximal intestine; a longer, wider and darker portion called distal intestine which ends at the anus [12].

Because the interest on IMs is quite recent, and although there is already a broad variety of studies on insect-fed fish focusing on different aspects of the intestine ([128,144-147], there is still work to be conducted in this area. These studies usually cover histomorphological parameters related in some way to the density/functionality of the absorption surface, the inflammatory status of the intestine or other parameters that could modify indirectly the intestinal function (width of different layers, villi height and width, density of supranuclear vacuoles or enterocyte height). Among all of them, villi height is the most commonly studied parameter, probably due to its obvious relationship with the absorption of nutrients and digestibility [148,149]. Many insect-fed fish studies describe little or no changes within the intestine structure [145,150-152], but there are also some studies that describe a reduction of villi height [136,144,153], or even an increase of this parameter [154]. Chitin usually acts as a "jack of all trades" to justify changes during insect-fed fish trials, which is not necessarily wrong due to the peculiarities of this molecule and its metabolites. However, the complex composition of IMs gives a generous amount of possibilities to elaborate about these changes, like one study which noticed an increase in villi height of mirror carp (*Cyprinus carpio* var. specularis) by using insect fat, not complete IM [147]. As usual, HI as the target insect is the most common among this kind of studies.

Microbiota study, and especially GIT microbiota, is a field of work with a big potential which, similarly to the use of IMs, has brought the attention of many researchers of the insect-fed fish area in the past few years [118,136,155]. The GIT of farmed fish is constantly in contact with rearing water and feed, generating a dynamic ecosystem rich in nutrients, and therefore favourable for the growth of the majority of bacteria [156-159]. On this basis, the perpetual state of gut colonization turns this variable into a very hard one to keep under control, which is why studies

that are apparently similar can lead to very different results [160-162]. In general, it is well accepted that the composition and abundance of the GIT microbiota plays an important role in nutrient absorption and protection against GIT infections. This could affect the growth performance, immune response, welfare status, and general health of the fish [159,163,164]. It is also known that autochthonous microorganisms (adhered to the intestine) are not the same as allochthonous microorganisms (transient, adhered to the digesta) [155,161]. The different phase of the insects used for feeding can also modify the intestinal microbiota [162], which makes the interpretation of data even more complicated. However, several studies on insect-fed fish have described small coincidences among them, like an increase in operational taxonomic units (OTUs) related to lactic acid bacteria and other probiotic microorganisms [115,155,162]. This could be due to the presence of chitin, its metabolites, or insect fat, which could act as high-affinity substrates for these bacteria.

5.4.4. Liver function: intermediary metabolism and liver histology

Once the nutrients have been absorbed and reach the bloodstream, they suffer several metabolic changes before they are integrated as part of the body mass. The intermediary metabolism is regulated by a broad battery of enzymes that are complexly interconnected among them. For example, glutamate dehydrogenase (GDH, EC 1.4.1.2), glutamate pyruvate transaminase (GPT, EC 2.6.1.2) and glutamate oxaloacetate transaminase (GOT, EC 2.6.1.1) are known to be good indicators of the AA metabolism, since their activities have shown increases and decreases when different levels of protein are included in the fish diet [165-167]. Similarly, pyruvate kinase (PK, EC 2.7.1.40) and fructose 1,6-bisphosphatase (FBP_{ase}, EC 3.1.3.11) are, respectively, good indicators of glycolytic and gluconeogenic processes [165,166,168]. Most nutritional experiments focused on the research of one ingredient should use isolipidic and isoproteic diets to standardise as many variables as possible. For this reason, it is no surprise that major changes have not been described in the behaviour of these enzymes for experiments with IMs [116,118,167,169], beyond the inherent differences among species [109]. However, the different nature of IM protein and fat could have minor repercussions in fish metabolism, which is why studying these variables could be interesting in the long term to keep optimising fish culture protocols and diets.

From a different perspective, morphological and histological studies can also provide insight on the status of liver physiology and fish metabolism. It is known that the size of the liver can change with variations in fish nutrition. An increase in hepatosomatic index can be due to liver lipidic accumulation, through lipid mobilisation [170-172]. A decrease in this same variable can be caused by prolonged starvation [173,174]. Actually, when this kind of analyses was carried out for insect-fed fish studies, different results were described, possibly due to the fish species that was studied [144,175,176]. Taking aside these exceptions, other liver-focused studies with insect-fed fish have only described minor or non-existent changes in the histology of this organ [152,154,177].

5.4.5. Animal welfare indicators: antioxidant response, immune system and otherplasma metabolites

During the last decade, the welfare of production animals has grown to become a real concern in society. Going further than fulfilling environmental/culture necessities of the fish or improving the different handling techniques and protocols, nutrition can also play an important role in this topic. The final objective of animal welfare measures is to prevent the appearance of unnecessary stress. The antioxidant response, the immune system and other indicators that are closely related to animal welfare are the most evaluated variables when it comes to studying this field of research.

The antioxidant response could be defined as a battery of molecules and enzymes that have the objective of neutralising harmful reactive oxygen species, which are naturally produced during the metabolism of living beings. Typically, the functionality of this system is evaluated through the activity of some among those same enzymes, like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH). Another way of performing this evaluation is through the consequences of oxidative stress and its damage, malondialdehyde (MDA) being a good indicator of lipidic peroxidation. There is a wide evidence that IMs can reduce the oxidative stress of different fish species [89,178-180], but little is still known about how it happens. It has been described that chitin, one of the main components of IMs, might act as a natural scavenger of reactive oxygen species at the same time that it can have an effect modulating the intracellular concentration of glutathione [181-183]. However, there are also evidences that other insect components such as insect oil may have similar effects by themselves [147,184].

The immune system is an extremely complex group of molecules and cells that serve the purpose of modulating and executing the inflammatory response to fight, mostly, microscopic external aggressions like infectious agents and parasites. Due precisely to its very high complexity, this system is normally studied from one of two ways: the adaptative response, or the innate response, which is the one that was evaluated for the purposes of this document. The innate or nonspecific response covers the functionality of all those molecules and cells that act as the first barrier against pathogens. Some examples of this would be nonspecific enzymes such as lysozymes or peroxidases, or even more specific molecules like immunoglobulins. Similar to the case of the antioxidant response, there is extensive evidence that IMs can enhance the immunological response in different ways, for both fish and crustaceans [89,185-187]. Chitin also appears as one of the most typical hypotheses to justify this enhancement, but some manuscripts assign these properties to other insect-related agents like antimicrobial peptides that have been described in the composition of different insects [188,189], insect frass, sometimes as a direct ingredient [190], or even the different composition of insect fat [147,152]. HI is very rich in lauric acid, a saturated FA with known antimicrobial activity [189,191]. The complex composition of IMs and the immune system makes it clear that there are still big mysteries to be solved on the topic. This, together with the previously mentioned, leads to the obvious conclusion that there will not be a truly effective way of using these ingredients as functional until the specific biochemical mechanisms underneath are properly isolated and identified.

Due to their close relation with the activation of the hypothalamo-pituitary-interrenal axis after a stressful situation [192], the levels of plasmatic glucose or lactate are also useful to evaluate the welfare status of fish [193,194]. These parameters could, but are not necessarily linked to, the effects of IMs, and there are not many studies describing important changes for these parameters on the context of insect-fed fish [195,196]. However, since these analyses are usually economic and easy to carry out, they can act as an interesting secondary method to control the status of the fish.

5.5. Effect on the quality of the final product

Due to its contribution as a supplier of essential n-3 and easily digestible protein of high value, fish play a crucial role in the correction of unbalanced diets and the production of a healthy one. Also, nutritional value, organoleptic properties and freshness are groups of characteristics that determine the quality of the fish as perceived by the consumer, and the final quality of the product refers to how the edible part of the fish can satisfy the market needs. This is why analysing these parameters is always interesting.

5.5.1. Fillet proximate and fatty acids compositions

The proximate composition of fish fillets can vary strongly due to external causes like the diet [70,72,197,198], and may ultimately affect the market acceptability [198]. Depending on the component that is being analysed, different effects can be described. For example, it is known that lipids play an important role on the edible part of the fish, especially in those species that do not store high amounts of fat within the muscle [199]. Changes in sensorial traits have been highlighted associated to an alteration of fillet lipids [200,201]. Similarly, differences in the perception of final products have been described when the levels of protein changed [202]. Moisture seems to be a more complicated parameter on this topic. Some manuscripts described changes in organoleptic or visual traits while a change in moisture occurred [203,204]. However, these changes in moisture are usually associated with fluctuations in other parameters that are known to be relevant, such as the levels of fat [198,201], or different events during the processing of food [203,205]. As such, it is complicated to establish if changes in moisture are directly as relevant as changes in other parameters.

Several insect-fed fish studies have described differences, either up or down, in the fat content of the fish or their fillets [90,104,178,206], but not as many differences in the levels of protein, ash or moisture [113,114,207]. Most insect-fed fish studies use larvae to manufacture diets, which usually have higher levels of fat than the adults, even though it depends on the species. For example, it has been described that HI can show levels of fat as high as 28 % during the early prepupa phase, and from 21.6 % to 32.2 % for adults [208], while TM larvae showed 32.7 % of fat, and a 7.6 % for adults [209]. This is one of the main reasons why whole insects act as limiting factors during their incorporation as ingredients in feed formulations. For the case of aquafeeds, the inclusion of an ingredient as protein source that also has a relevant amount of fat in its composition implies a decrease in the fish oil inclusion, and therefore changes the nature of the fat. In this regard, the FA profile of fish oil is known for having a very rich concentration of LC n-3 PUFA, and especially eicosapentaenoic, docosapentaenoic or docosahexaenoic acids, which are hard to find in land-based components. LC n-3 PUFA are known for having very

positive effects towards the prevention of cardiovascular diseases in human health, and their functionality can be evaluated with different indices that compare the proportion of different FAs, such as the thrombogenic index (TI), atherogenic index (AI), omega-6/omega-3 ratio (n-6/n-3), and/or fish lipid quality index (FLQ) of a particular foodstuff [210-212].

Insect fat of the most studied insects have a drastically different FA profile when compared to fish. Their FA profile is characterised by a higher proportion of short chain, monounsaturated, and n-6 FAs such as palmitic and oleic acids, the case of LC n-3 PUFA being almost non-existent [70,72]. It would also be worthwhile to mention that different insects have relevant interspecific differences in their FA profile. For example, and as stated in a previous point, HI has an elevated proportion of lauric acid in its composition, while TM has very high levels of both oleic and linoleic acids [70,72]. Adding all the above mentioned together, this acts as a weak point for insect-fed fish. It has been reported in several manuscripts that these fish have their LC n-3 PUFA lowered in a consistent way [73,113,213-216], weakening one of the most attractive aspects of eating fish as a main source of these FAs. However, it should be possible to correct this problem through different strategies, like the addition of a LC n-3 PUFA rich ingredient in the fish diet [217], the enrichment of the fat fraction of insects through their diet [74,104,218], or the reduction of the contribution of this fat fraction to the feeding formula, through a defatting process of the IM [219-221].

5.5.2. Instrumental and sensorial parameters of the fillet

After all the physiological/nutritional analyses, in the end, any new product needs to achieve a common goal in order to be viable: it must be acceptable from the perspective of the target consumer.

The objective characteristics of the fillets, such as the texture and/or colour that can be analysed with different instruments can be affected through the fish diet [222,223]. The case of the subjective perception, like the one that can be analysed through direct sensorial analyses, is not different, especially when the amount or the nature of the fat is modified [223-225]. For these reasons, and because IMs are novel ingredients, it is important to discover if they can modify the perception of the fillet after an insect-fed fish trial.

So far, and even though it is not so frequent to find experiments where the perception of the fillets was tested after an insect-fed fish trial, most of them tend to agree on the fact that IMs do not modify these traits in a significant way [104,219,226,227]. One experiment described relevant changes in parameters related to the sensorial profile of insect-fed fish [228]. The most common conclusion, however, is that IMs only modify the colour of the fillet and/or the skin of the fish [229-231], possibly due to the different nature of the fat. Another feasible possibility for experiments with TM would be the high levels of riboflavin (vitamin B2) in this insect [232], a yellow-coloured pigment that might contribute to these changes in colour.

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CHAPTER 2 – Hypothesis and objectives

HYPOTHESIS AND OBJECTIVES

Hypothesis: insect meals can be used as an alternative source of protein as a partial replacement of fishmeal. Their inclusion level in diets for rainbow trout is determined by their composition, which will depend on the insect species. The maximum acceptable inclusion level will also depend on the performance of growth performance, fish physiology and final quality of the fillets.

General objective: The present thesis has the goal of providing insight on the partial replacement of fishmeal using insect meals (particularly, black soldier fly, *Hermetia illucens* and yellow mealworm, *Tenebrio molitor*) for the feed formulations of rainbow trout (*Oncorhynchus mykiss)*.

Specific objectives:

1) Assay different fishmeal replacement levels with insect meal to find optimal proportions for both insect meals in rainbow trout feed formulations. [Studies 1 and 2]

2) Study the growth performance of insect-fed rainbow trout to discover if there are significant differences between the two used insects, including other variables directly related to growth such as protein use and biometric indices. [Studies 1 and 2]

3) Study other physiological repercussions that might happen and/or be relevant during an insect-fed fish experience. For that, different variables are measured such as: digestive enzymes, intestinal health (histomorphology and microbiota), liver function (intermediary metabolism and histomorphology) and several animal welfare indicators (antioxidant response, immunological system and other plasmatic metabolites). [Studies 1, 2 and 3]

4) Study the possible repercussions of feeding rainbow trout with insects on the quality of the final product, implying the analysis of fillet composition (proximal and fatty acids) and the repercussion on the perspective of the target consumer. For that, different characteristics were measured in both the instrumental (colour and texture) and perceived (direct raw and cooked sensorial analyses) quality of the fillets. [Studies 1 and 3]

5) Assay different feed formulation strategies to compensate the decrease of long chain omega-3 polyunsaturated fatty acids that typically occurs during insect-fed fish experiments. [Study 3]

6) Study if any of the objectives previously mentioned are particularly influenced by the developmental stage of rainbow trout. [Studies 1, 2 and 3]

CHAPTER 3 – Study 1

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ORIGINAL ARTICLE

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Potential use of black soldier fly (*Hermetia illucens*) and mealworm (*Tenebrio molitor*) insectmeals in diets for rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The aquaculture industry is diminishing the amount of fishmeal needed to maintain its protein demand. Alternatives are tested in this way, being insects one of the most promising. In this study, two different insectmeals were tested at different fishmeal replacement proportions (150 and 300 g·kg^{-1}) in diets for *Oncorhynchus mykiss*. This study covers diverse aspects related to growth, protein utilization, physiological status of the fish, and quality of the final product. The inclusion of insectmeals had no negative effects on growth, protein utilization and on the physiological status of the fish. At the highest fishmeal replacement level with *Tenebrio molitor*, fish showed a higher digestibility of the protein, a reduction in viscerosomatic index and a higher aerobic catabolism, generating a pro-oxidative environment that was compensated by an increase in antioxidant enzymes, revealing the importance of choosing the appropriate insectmeal. A significant reduction in omega-3 fatty acids in the fillet was observed with an increasing insectmeal inclusion. The study proves that insectmeal can be a viable alternative for the partial replacement of fishmeal in rainbow trout, but further studies are needed to determine the most appropriate insectmeal, and to deal with the reduction of omega-3 fatty acids.

KEYWORDS
Black soldier fly, fatty acids, fishmeal replacement, insectmeal, mealworm, rainbow trout

1 | INTRODUCTION

Global population is in constant growth, and so is its global protein demand. Aquaculture has been highlighted as one of the best possibilities to satisfy animal protein demand due to the remarkable efficiency of fish growth in comparison with terrestrial animals (Fry, 2018), and to its fast development as an industrial food sector. The most commonly farmed fish require aquafeeds with a high content in protein, whose demand has been traditionally satisfied with a significant inclusion of fishmeal (FM) in fish feeding formulations. In order to favour this continuous development in a sustainable way, the scientific and industrial communities have invested big efforts on

reducing the inclusion of FM by searching for alternative ingredients (Gasco, 2020; Mousavi, 2020; Tran, 2015). Nonetheless, and even though it has been proven that it is possible to drastically reduce the amount of FM in fish feedings (Motte, 2019; Stenberg, 2019) the increase of FM overall absolute consumption is expected to keep increasing until 2030 together with the growth of aquaculture (FAO, 2020). With this increase come two main problems: the ecological impact of using high quantities of harvested wild sea fish for this objective, and the consequential increase of FM economic value.

The search for viable alternatives to FM continues to be a main challenge. In this sense, the use of insectmeals (IM) as sources of protein is one of the most promising options (Hua, 2019; Tran, 2015). First, they have shown a big potential as a sustainable animal protein source, due to the facts that they grow and reproduce easily, their growth ratios are very efficient, and their production requires low resources regarding to space and energy investment (van Huis, 2013). Also, there is a wide bibliography that proves its promising results in different fish species. In *Sparus aurata*, Piccolo (2017) achieved the highest growth performance at 330 g·kg^{-1} FM replacement with *Tenebrio molitor* (TM) in feed formulation. Magalhães (2017) highlighted that *Hermetia illucens* (HI) meal may successfully replace up to 195 g·kg^{-1} FM in diets for juvenile *Dicentrarchus labrax* without adverse effects on growth performance. Ji (2015) also expanded this line of work by demonstrating that even at high levels of FM replacement (500–600 g·kg^{-1}) with silkworm meal (*Bombyx mori*), a species such as Jian carp (*Cyprinus carpio* var. Jian) can maintain growth ratios similar to those which could be seen with no replacement.

More specifically for salmonids, different levels of FM replacement with different IMs have been tried. As examples of not impairing growth, Terova (2019) replaced up to 300 g·kg^{-1} FM with HI in rainbow trout. Belghit (2019) described the possibility of completely replacing FM for Atlantic salmon (*Salmo salar*), using HI as one of the chosen protein sources. With a slightly different approach, the work of Mikołajczak (2020) tested a 400 g·kg^{-1} replacement of FM with two hydrolysed IMs (TM, and superworm, *Zophobas morio*) on sea trout fingerlings (*Salmo trutta*), and only noticed a change in protein efficiency ratio. Similarly, a recent trial of Chemello (2020) noted no changes in growth for rainbow trout with a complete substitution of FM, in this case, using TM as a main source of protein. According to these and other published data, it is quite easy to assume that, with little differences among species, but in general terms, a partial replacement of FM with IM has no negative effects on the growth of most fish species, especially when there is more than one main protein source involved.

The different composition of the diets, and therefore different sources of protein, may affect the physiological status of fish. The use of IM has been related with differences in digestive enzyme activities (Rapatsa & Moyo, 2017, 2019), changes in liver metabolic enzymes (Chaklader, 2019), and an increase in the antioxidant capacity (Li, 2017; Taufek, 2016). Following this line of knowledge, this work had the objective of helping clarify the impact of the partial replacement of FM by IM in the feeding formulation of rainbow trout (*Oncorhynchus mykiss*). Two different insect species, black soldier fly (HI) and mealworm (TM), at two different FM replacement levels (150 and 300 g·kg^{-1}) were used, and the effect on different parameters related to growth efficiency, physiological status and final quality of the fish were evaluated, to try to elucidate the nutritional feasibility of both IM in feed for rainbow trout.

2 | MATERIAL AND METHODS

2.1 | Experimental diets

Commercial insectmeal (IM) from two different species, *Hermetia illucens* (HI) and *Tenebrio molitor* (TM; Mealfood Europe S.L., Spain) in larvae stage were used in this study. Before feed formulation, IM were analysed (Table 1). In this study, a total of five isoproteic (460 g·kg^{-1}) and isolipidic (170 g·kg^{-1}) diets were formulated (Table 2), a control diet with no IM, and four diets with two replacement levels and two sources of IM: H1 and T1 had 150 g·kg^{-1} FM replacement, and H2 and T2 had 300 g·kg^{-1} FM replacement. Ingredients were provided by 'Lorca Nutrición Animal S.A.' (Murcia, Spain). Diets were supplemented with methionine and phenylalanine to address the nutritional requirements of rainbow trout (Blanco Cachafeiro, 1995; National Research Council, 1993), manufactured by LifeBIOENCAPSULATION S.L. (Almería, Spain), and extruded in 2 mm pellets. The dough was passed through a single screw laboratory extruder (Miltenz 51SP, JSConwell Ltd, New Zealand). The extruder barrel consisted of four sections and the temperature profile in each section (from inlet to outlet) was 100 ºC, 95 ºC, 90 ºC and 85 ºC, respectively. Finally, pellets were dried in a drying chamber at 30 ºC for 24 hr (Airfrio, Spain) and kept in sealed plastic bags at −20°C until use.

2.2 | Experimental animals and rearing conditions

600 female rainbow trouts with an initial weight of 55 ± 0.7 g were acquired from a commercial farm (Piscifactoría Fuente del Campillo, Guadalajara, Spain), and transported to the experimental facilities of the Aquaculture Research Centre of "Instituto Tecnológico Agrario de Castilla y León" (ITACyL). Fish were held without treatment for acclimation during 15 days before growth trial, and then, they were randomly allocated in groups of 30 animals into 20 cylindrical fiberglass tanks (500 L) in a recirculating system. Diets were assayed in quadruplicate. Fish were hand-fed to apparent satiation once a day (9 a.m., up to a maximum of 3.100 g fish^{-1}·day^{-1} feed intake) for 46 days. During the growth trial, water temperature was maintained at 15 ± 1°C, dissolved oxygen at 7.6 ± 1 mg·L^{-1}, and the photoperiod was 12 hr light:12 hr dark. The concentration of ammonia and nitrite in water were monitored daily to make sure that they were at optimal levels (ammonia < 0.1 mg/L and nitrite < 0.1 mg/L).

All procedures for the care and handling of rainbow trout were conducted in accordance with specific regulations, the Directive of the European Union Council (2010/63/EU) and the Spanish

TABLE 1 Composition of insectmeals from *Tenebrio molitor* and *Hermetia illucens* used in the experiment

Proximate composition (g·kg^{-1} insectmeal)	*Hermetia illucens*	*Tenebrio molitor*
Crude protein	300.0	420.2
Crude fat	339.2	283.4
Crude ash	105.9	36.8
Moisture	32.2	53.4
Phosphorus	6	7.1
Calcium	48	1.2
Chitin	165.7	58.7
Fatty acid composition (g·kg^{-1} fatty acids)		
C10:0 (*capric acid*)	11.89	0
C12:0 (*lauric acid*)	429.1	0
C14:0 (*myristic acid*)	83.3	23.9
C16:0 (*palmitic acid*)	147.4	178.1
C16:1n7 (*palmitoleic acid*)	24.4	14.1
C18:0 (*stearic acid*)	26.9	34.8
C18:1n9 (*oleic acid*)	153.5	345.7
C18:2n6 (*linoleic acid*)	88.7	374.7
C18:3n3 (*α-linolenic acid*)	5.7	17
C18:4n3 (*stearidonic acid*)	9.79	0
Amino acid composition (g·kg^{-1} insectmeal)		
Asp (aspartate)	26.8	33.4
Thr (threonine)	7.7	12.4
Ser (serine)	12.1	20.8
Glu (glutamate)	28.3	48.2
Pro (proline)	13.9	29.6
Gly (glycine)	15.8	23.2
Ala (alanine)	22.7	34.0
Val (valine)	12.2	18.7
Met (methionine)	4.0	5.1
Ile (isoleucine)	8.0	12.2
Leu (leucine)	16.6	25.3
Tyr (tyrosine)	14.1	23.9
Phe (phenylalanine)	10.8	15.3
His (histidine)	6.6	13.2
Lys (lysine)	13.8	21.3
Arg (arginine)	10.5	19.3

Government (Real Decreto 53/2013), and previously approved by the Bioethical Committee of the "ITACyL" (Authorization number: 2017/19/CEEA).

2.3 | Growth trial and sampling collection

During the experiment, feed intake and mortality were monitored, and all fish were weighed each 21 days to monitor their growth. In order to achieve that, after fasting for one day, fish were anesthetized with tricaine methanesulfonate (MS-222;180 mg·mL^{-1}), and body length and weight were measured using a graduated ictiometer (± 0.1 mm) and scale (± 0.1 g), respectively. To analyse apparent digestibility, faeces were collected during the last two weeks of the growth trial by a modified Guelph method (Cho, 1982), gathering the faeces produced throughout 24 hr in a settling column. The faeces samples were frozen at −80°C until their analysis.

At the end of the experiment, two fish were randomly sampled from each tank (8 fish per diet), and sacrificed by an overdose of MS-222 (300 mg·mL^{-1}). Blood, liver, stomach, intestine with pyloric ceca, and fillet samples were collected and individually analysed.

Blood samples were collected with heparinized syringes and its plasma separated by centrifugation at 3,500 x g for 15 min at 4 ºC. Individual plasma samples were frozen at −80 ºC until their analysis.

For enzyme determinations the tissue samples were immediately frozen in liquid nitrogen and maintained at −80 ºC until their analysis. For chemical analysis and fatty acid (FA) determinations, the samples were directly frozen at −80 ºC until their analysis.

2.4 | Analytical determinations

2.4.1 | Chemical analysis

Insect meal, diets and fish fillets were analysed for fat content, moisture according to AOAC methods (2005), and crude protein content was analysed according to Dumas method (Saint-Denis & Goupy, 2004) in a nitrogen analyser (FP 528, LECO, St. Joseph, USA). The conversion factor for protein content determination was 6.25 for feeds and faeces, and 4.67 for HI and 4.75 for TM (Janssen, 2017).

The apparent digestibility of the protein was determined using acid-insoluble ash as marker in feeds and faeces (Atkinson et al., 1984).

In the case of insect meal, phosphorus (P) was determined by molecular absorption spectrophotometry according to ISO standard (1996) using a spectrophotometer (UV/Vis UV2, UNICAM, Cambridge, UK). For the determination of calcium (Ca) X-ray fluorescence method of Dispersive Energy (ED-XRF) was used, based on the methodology described by Pessoa (2016). Chitin was isolated from insect meal using a methodology described by Gamage and Shahidi (2007). The obtained chitin residue was washed with acetone, dried and weighed.

For the amino acids, 1.6 and 1.5 mg of IM samples (*T. molitor* and *H. illucens*, respectively), were hydrolysed with 200 μl of 6 N HCl for 22 hr at 110°C, and the determination was performed by ion-exchange liquid chromatography and postcolumn continuous reaction with ninhydrin (Biochrom 30; United Kingdom) to provide qualitative and quantitative compositional analysis.

2.4.2 | Fatty acids determination

FA analyses in IMs and fish fillets were carried out as in previous studies (Lepage & Roy, 1984; Rodríguez Ruiz et al., 1998). FA were

TABLE 2 Ingredients, proximate and fatty acids composition of experimental diets

Ingredients (g·kg^{-1} on wet basis)	C	H1	H2	T1	T2
Fishmeal	368	315.4	259.1	313.3	258.9
HI meal	0.0	56.3	109.1	0.0	0.0
TM meal	0.0	0.0	0.0	51.3	102.7
Wheat gluten	105.3	127.3	148.3	120.9	126.1
Soy protein concentrate	150.9	158.6	170.2	150.7	165.3
Wheat meal	162	139.5	119.2	156	146.3
Soy lecithin	12.7	9.7	9.7	9.7	7.8
Fish oil	116.8	108	98.3	112.8	106.9
Vitamins and minerals	19.5	19.5	19.5	19.5	19.4
Goma guar	19.5	19.5	19.5	19.5	19.4
Hemoglobin	38.9	38.9	38.9	38.9	38.9
Methionine	0.8	1.5	2.2	1.5	2.2
Lysine	5.6	5.8	6.0	5.9	6.1
Total	**1,000**	**1,000**	**1,000**	**1,000**	**1,000**
Proximate composition (g·kg^{-1})					
Crude protein	458.1	466.2	454.3	465.4	463.1
Crude fat	164.6	174.1	173.2	174.2	167.7
Crude fiber	15.2	18.9	14.7	13.1	19.0
Ash	81.0	79.2	77.8	74.3	81.8
Fatty acid composition (g·kg^{-1} fatty acids)					
C12:0 (*lauric*)	0	52.2	96	0	0
C14:0 (*myristic*)	49.5	52	54.4	46.4	44.6
C16:0 (*palmitic*)	193.1	179.2	178	187.1	190.2
C16:1n7 (*palmitoleic*)	50	46.9	44.2	47.4	44.1
C17:0 (*margaric*)	7.76	6.90	6.08	7.13	6.46
C18:0 (*stearic*)	40.8	37.1	35.6	38.9	39.1
C18:1n9 (*oleic*)	143.8	145.6	145.9	163.3	181.5
C18:1n7 (*vaccenic*)	27.7	24.6	22.6	25.6	23
C18:2n6 (*linoleic*)	93.9	96.4	106.8	120.6	149.7
C18:3n3 (*linolenic*)	16.36	15.2	14.9	16.4	16.7
C18:4n3 (*stearidonic*)	15.1	13.4	11.6	13.9	12.5
C20:1n9 (*eicosenoic*)	30.3	28.2	22.4	26.6	23.7
C20:4n6 (*arachidonic*)	10.2	9.18	8.12	9.42	8.45
C20:4n3 (*eicosatetraenoic*)	5.45	4.87	4.31	5.04	4.56
C20:5n3 *(eicosapentaenoic; EPA)*	88.9	79.5	69.7	82.4	74.2
C22:1n11 (*cetoleic*)	37.1	33.4	29.3	34.7	30.9
C22:5n3 *(docosapentaenoic; DPA)*	13.4	11.6	9.87	12	10.8
C22:6n3 (*docosahexaenoic; DHA)*	118.4	106.1	90	109.1	96.8
C24:1n9 (*nervonic*)	4.56	3.89	3.29	4.04	3.57
SFA (*saturated fatty acids*)	291.2	327.4	370.1	279.5	280.4
MUFA (*monounsaturated fatty acids*)	293.4	282.5	267.7	301.5	306.9
PUFA (*polyunsaturated fatty acids*)	361.7	336.2	315.4	368.9	373.6
n−3	257.6	230.6	200.5	238.8	215.5
n−6	104	105.6	114.9	130.1	158.1
n−6/n−3	0.40	0.46	0.57	0.54	0.73

(Continues)

TABLE 2 (Continued)

Note: Vitamin and mineral premix (g·kg^{-1} unless otherwise specified): vitamin A 2,000,000 UI; vitamin D3: 200,000 UI; vitamin E: 12; vitamin K3: 2.6; vitamin B1: 3; vitamin B2: 3; vitamin B6: 2; vitamin B9:1.5; vitamin B12: 0.01; vitamin H: 0.3; inositol: 50; betaine: 50; calcium pantothenate: 10; nicotic acid: 20; Co: 0.06; Cu: 0.9; Fe: 0.6; I: 0.05; Mn: 0.95; Se: 0.001; Zn: 0.75; Ca: 190; K: 24; Na: 41.C: control diet (no insectmeal inclusion); H1 and H2: 150 and 300 g·kg^{-1} fishmeal replacement with Hermetia illucens (HI), respectively; T1 and T2: 150 and 300 g·kg^{-1} fishmeal replacement with Tenebrio molitor (TM) respectively. n−6/n−3 is a non-dimensional ratio.

measured after direct derivatization to FA methyl esters (FAME). FAME were analysed in a Focus GC (Thermo Electron. Cambridge. UK) equipped with a flame ionisation detector (FID) and an Omegawax capillary column (30 m x 0.25 mm i.d. x 0.25 μm film thickness; Supelco. Bellefonte. USA), as previously described (Guil-Guerrero, 2014). Peaks were identified by retention times obtained for known FAME standards (PUFAs No. 1, 47,033 from Sigma, St. Louis, MO, USA), and FA contents were estimated by using methyl pentadecanoate (15:0; 995 g·kg^{-1} purity; 76,560 Fluka) from Sigma as internal standard. The relative retention factors for each FA were estimated as reported by Cladis (2014) were considered for quantification.

2.4.3 | Digestive enzymes determination

For digestive enzymes, stomach, and intestine with pyloric ceca were separately processed. Individual digestive samples were first homogenized with distilled water (250 mg·mL^{-1}) at 4°C. Stomach extracts were used to measure acid protease activity, and extracts of intestine with pyloric ceca were used to measure amylase and alkaline protease activities. The concentration of soluble protein in samples was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific TM, Rockford, IL) using bovine serum albumin as a standard. Amylase activity was determined according to the Somogy-Nelson method (Somogyi, 1952) using soluble starch 20 g·kg^{-1} as substrate. One unit of activity was defined as the amount of enzyme able to produce 1 μg of maltose per minute and mg of protein. Alkaline protease activity was measured by Walter method (1984) using casein 10 g·kg^{-1} as substrate. Acid protease activity was measured by Anson method (1938), using hemoglobin 5 g·kg^{-1} as substrate. One unit of activity for both proteases was defined as 1 μg of tyrosine released per minute and mg of protein. All digestive enzyme activity analysis were performed at 37 ºC.

2.4.4 | Intermediary metabolism and antioxidant status

Liver samples were individually homogenized in nine volumes of ice-cold 100 mM Tris-HCl buffer containing 0.1 mM EDTA and 1 g·kg^{-1} (v/v) Triton X-100, pH 7.8. All procedures were performed on ice. Homogenates were centrifuged at 30,000 x g for 30 min at 4°C and the resultant supernatants were kept in aliquots and stored at −80°C for further enzyme assays.

Fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11), glycerol kinase (GyK; EC 2.7.1.30), pyruvate kinase (PK, EC 2.7.1.40), lactate dehydrogenase (LDH, EC 1.1.1.27), citrate synthase (CS; EC 4.1.3.7), β-hydroxyacyl CoA dehydrogenase (HOAD; EC 1.1.1.35), glutamate pyruvate transaminase (GPT; EC 2.6.1.2), glutamate oxaloacetate transaminase (GOT; EC 2.6.1.1), and glutamate dehydrogenase (GDH; EC 1.4.1.2) were determined as previously described by Furné (2012).

Superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), and glutathione reductase (GR, EC 1.6.4.2) were determined according to Pérez-Jiménez (2009).

All enzyme assays were carried out at 25°C and changes in absorbance were monitored to determine the enzyme activity using a PowerWaveX microplate scanning spectrophotometer (Bio-Tek Instruments, USA). The optimal substrate and protein concentrations for the measurement of maximal activity for each enzyme were established by preliminary assays. The millimolar extinction coefficients used for NADH/NADPH, DTNB, and H_2O_2 were 6.22, 13.6, and 0.039·mM^{-1}·cm^{-1}, respectively. One unit of SOD activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome C reduction rate. For the rest of enzymes, one unit of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate per min. Lipid peroxidation was determined based on malondialdehyde (MDA) levels. In the presence of thiobarbituric acid, MDA reacts producing coloured thiobarbituric acid reacting substances (TBARS) that were measured as previously described by Pérez-Jiménez (2009).

Soluble protein concentration in tissue homogenates was analysed using the method of Bradford (1976), with bovine serum albumin used as a standard.

2.4.5 | Non-specific immune status

Plasma lysozyme activity was assayed according to Swain (2007), using a turbidometric method with *Micrococcus lysodeikticus* (Sigma, St. Louis, USA). For the standard curve hen egg white lysozyme was used. The reaction run for 20 min at 35°C and measured at 450 nm.

Carbonic anhydrase has esterase activity. This plasma activity was assayed according to Mashiter and Morgan (1975). For this, the total esterase activity was measured at 25 ºC, using 0.8 mM p-nitrophenyl acetate as a substrate, and subtracting the activity of the rest of the esterases except the carbonic anhydrase that is inhibited, using 1.6 mM acetazolamide as an inhibitor. After an incubation period of 10 min for each of the reactions, the absorbance increase at 405 nm was measured for 5 min.

A trypsin assay in absence of plasma was used as control to measure antiprotease activity according to the method of Thompson (1995). The production of 4-nitroaniline was determined by the variation of the OD (optical density) at 410 nm for 30 min. Trypsin activity was used as a control (CAS 90002–07–7, Acofarma, Spain).

Phosphatase activity was measured using p-nitrophenil phosphate (Sigma) as substrate. For this, a buffer at pH 10 ($NaHCO_3$/NaOH 0.05 M, $MgCl_2$ 1 mM) was used to measure alkaline phosphatase activity and a buffer at pH 5 (CH_3COOH/CH_3COONa 0.1 M, $MgCl_2$ 1 mM) for acid phosphatase activity. The measurement was performed for 30 min, at 37°C and at 405 nm (Huang, 2011).

Peroxidase activity in plasma was assayed according to Mohanty and Sahoo (2010), using a solution 20 mM TMB (3, 3‵, 5, 5‵-Tetramethylbenzidine) as substrate. The colour change reaction was stopped after 2 min; then samples were read at 450 nm. Plasma-free standard samples were measured as controls. The activity was expressed in OD (optical density).

Total immunoglobulin in plasma was determined as described by Panigrahi (2005). Immunoglobulins were separated from the total proteins by precipitation with polyethylene glycol. Supernatant total protein was determined by Bradford method (Bradford, 1976). Total immunoglobulin content was determined by subtracting the protein content resulting from the total protein content in the untreated plasma.

2.5 | Statistical analysis

SAS system version 9.0 (SAS Institute Inc., Cary, North Carolina, USA) was used for the statistical analysis. Since all the variables fit normal distribution, the data were analysed using a general linear model (PROC GLM) analyses of variance (ANOVA). The values are showed as mean ± standard error of the mean.

3 | RESULTS AND DISCUSSION

The approval of the use of insects as ingredients in aquafeeds by the European regulation (EU, 2017) has increased the interest in these ingredients by both manufacturers and scientists in the last years. The study aims to highlight the potential use of insectmeal (IM) as raw material in feeds for rainbow trout and the challenges that it faces, with the novelty of testing two different insect species (*Tenebrio molitor*, TM, and *Hermetia illucens*, HI) comparing different inclusion levels.

For the time being, the works carried out in rainbow trout involving the study of IMs have used animals between 5–545 g, with a replacement of 0–1000 $g \cdot kg^{-1}$ fishmeal (FM) by IM, during 56–152 feeding days. In particular, studies which included HI meal observed the tendency that this ingredient causes little to none changes in the normal physiology of rainbow trout (Dumas, 2018; Elia, 2018; Rimoldi, 2019; St-Hilaire, 2007). The most common change tends to be the reduction on growth parameters as the amount of replaced FM reaches 200–300 $g \cdot kg^{-1}$, as well as the reduction of omega-3 FA (*n-3*) on the fillet, which is due to the different composition of insect fat versus fish oil. Studies which used TM as replacement of FM in rainbow trout highlighted, in general, a better result in growth parameters, whilst the proportion of *n-3* in fillet tends to be even lower than the case of studies based on HI (Belforti, 2015; Borgogno, 2017; Iaconisi, 2017, 2018; Mancini, 2018; Renna, 2017; St-Hilaire, 2007). This difference between the performance of HI and TM could be due to their differences in composition, mostly aminoacids, calcium and chitin (Table 1).

The growth results displayed in this study were adequate for all diets. No mortality was recorded, and all diets were accepted correctly by the fish. The influence of the tested diets during the growth trial are shown in Table 3. There were no significant differences ($p > .05$) observed among the groups treated with different levels of IM inclusions, either for final body weight (FBW), specific growth rate (SGR) or daily feed intake (DFI) when comparing with the control diet. The FCR was adequate for all groups without differences respect to control diet, but it is worth to mention that the best FCR was showed for the fish fed with T1 diet, statistically different from H1 and H2 diets, which opens a possibility for future studies of improving the FCR through finding the optimal proportion and insect species in the feed formulation. Although most studies support the idea of a lesser proportion of FM (50–250 $g \cdot kg^{-1}$) replacement with IM not being beneficial or disruptive for fish growth (Iaconisi, 2017; Magalhães, 2017; Renna, 2017; Terova, 2019), there are other which uphold the possibility of a particular proportion of IM as beneficial for some fish species (Ido, 2015; Ng, 2001; Rema, 2019; Sing, 2014). However, when it comes to evaluate the effect of high levels of FM replacement with IM (650–1000 $g \cdot kg^{-1}$), the conclusions tend to diverge widely (Ido, 2019; Ji, 2015; Kroeckel, 2012; Lock, 2016; Vargas, 2018; Vargas-Abúndez, 2019). Similarly, when the protein utilization was evaluated, no differences were observed for protein efficiency ratio (PER) and productive protein ratio (PPV) among diets. As for apparent digestibility of the protein (ADC_{prot}), IM treatments showed higher levels, being the case of T2 as significantly different respect to control diet ($p < .05$; Table 3). In other words, the protein from IM included in the feed formulation showed a better availability for its utilization. Other authors found no differences in protein utilization in rainbow trout with the inclusion of HI (Dumas, 2018; Renna, 2017), and TM (Rema, 2019) up to 300 $g \cdot kg^{-1}$ in feed formulation. On the other hand, insects are part of the natural diet of rainbow trout (Metcalf, 1997; Rikardsen & Sandring, 2006); it is therefore not surprising that they are able to efficiently digest insect protein.

In relation to biometric indexes, non-negative effects were observed when the IM was included in the feed formulation (Table 3). However, it is remarkable that T2 had the lowest viscerosomatic index (VSI) value, significantly different to the rest of the diets. Changes in VSI can be related to changes in visceral fat deposition (Jobling, 1998, 2002), but a decreased VSI can also be related with a lower relative intestinal length. Since visceral fat deposition and relative intestinal length were not measured, these two indexes will be

TABLE 3 Effect of insectmeals in diets on growth performance, protein utilization and biometric indexes at the end of the feeding trial of rainbow trout

Growth performance	C	H1	H2	T1	T2	*SEM*
IBW (g)	55.4	54.9	56.5	53.7	56.0	0.7
FBW (g)	141.3	140.0	139.7	141.9	139.8	0.9
SGR (%·day^{-1})	2.04	2.02	2.02	2.05	2.02	0.01
DFI (g·100g fish^{-1}·day^{-1})	1.46	1.47	1.47	1.44	1.46	0.01
FCR	0.77^{ab}	0.78^{b}	0.78^{b}	0.75^{a}	0.77^{ab}	0.004
Protein utilization						
PER	2.85	2.85	2.84	2.86	2.85	0.03
PPV (%)	56.1	55.5	55.9	55	54.9	0.44
ADC_{prot} (%)	86.1^{a}	89.0^{ab}	88.7^{ab}	88.5^{ab}	90.6^{b}	0.9
Biometric indexes						
CF (g·cm^{-3})	1.13	1.15	1.14	1.15	1.12	0.01
HSI (%)	1.24	1.20	1.10	1.22	1.15	0.06
VSI (%)	9.58^{b}	10.16^{b}	9.87^{b}	9.88^{b}	8.82^{a}	0.25

Note: C: control diet (no insectmeal inclusion); H1 and H2: 150 and 300 g·kg^{-1} fishmeal replacement with Hermetia illucens, respectively; T1 and T2: 150 and 300 g·kg^{-1} fishmeal replacement with Tenebrio molitor respectively. IBW: initial body weight; FBW: final body weight; SGR (specific growth rate) = [(ln FBW-ln IBW)·days^{-1}] · 100; DFI (daily feed intake) = [daily feed consumption (g)·biomass^{-1} (g) at time] · 100; FCR (feed conversion ratio) = [total feed intake (g) · (FBW-IBW) $^{-1}$]; PER (protein efficiency ratio) = [total weight gain (g)·protein intake^{-1} (g)]; PPV (productive protein value) = [(protein gain (g) · protein intake^{-1} (g)) · 100]; ADC_{prot} (apparent digestibility coefficient of the protein) = 100-[(marker in diet (g)·marker in faeces^{-1}(g)) · (% protein in faeces·% protein in diet^{-1}) · 100]; CF (condition factor) = [weight (g)·length^{-3} (cm)] · 100; HIS (hepatosomatic index) = [wet liver weight·FBW^{-1}] · 100; VSI (viscerosomatic index) = [wet visceral weight·FBW^{-1}] · 100. $^{a, b}$ show statistically significant differences among diets ($p < .05$); Values are expressed as mean ± standard error of the mean (SEM; n = 4 tanks per diet, 2 fish per tank).

considered for further studies, as the present study reflects a promising match between a lower VSI and a higher apparent digestibility of the protein for T2.

The inclusion of new ingredients in feed formulation could also affect the physiological status of fish, and lastly, health and welfare status. For that, different parameters related to digestive enzyme activities, key metabolic hepatic enzymes, antioxidant and immune status were analysed.

The proteolytic and amylase activity may be different between fish with different nutritional habits (Hidalgo, 1999). Different authors have described differences in the activities of proteases between *Oreochromis mossambicus* and *Clarias gariepinus*, using the same diets with increasing amounts of *Imbrasia belina* meal (Rapatsa & Moyo, 2017, 2019). For that, the analysis of these digestive enzymes was included in this study, to evaluate the acceptation by rainbow trout, and to determine possible differences between the use of one insect or another (Table 4). The higher amount of alkaline protease observed in fish fed with TM matched with the increase in ADC_{prot} observed in these fish (Table 3), suggesting that intestinal proteases could be more relevant in the proteolysis and absorption of IM protein. There were no significant differences for acid proteases (Table 4; $p > .05$). Respect to amylase, a decrease in comparison to control diet is observed in the case of H2. Contrarily, Rapatsa and Moyo (2017, 2019) highlighted that higher levels of *Imbrasia belina* meal increased the levels of amylase; however, they did not suggest a direct relation between amylase and IM itself, but between amylase activity and the remaining vegetable contents of *Imbrasia belina*. Also, German (2004) reported that amylase activity increased with the quantity of carbohydrates in the diet. In this way, due to the lack of a direct relationship between IM and amylase function (starch hydrolysis) in the bibliography, and also the fact that there was only a significant difference between C and H2, this decrease should be due to the reduction of wheat meal in the formulation of this diet (a plant-based ingredient), since it was one of the ingredients that suffered the biggest reduction to adjust protein and lipids in HI diets but not in TM ones, being the reduction of wheat meal in H2 more important.

The liver is an essential metabolic organ which governs body energy metabolism. It acts as a hub to connect metabolically various tissues, including muscle and adipose tissue (Rui, 2014). The different composition of the diets may play an important role on the regulation of the enzymes involved in catabolism and anabolism of macronutrients. In this study, different key hepatic enzymes on metabolism were analysed (Table 5) to evaluate if the inclusion of IM in the diet could affect the fate of nutrients, either as part of a metabolic pathway or for energy storage.

Concerning lipid metabolism, HOAD (β-hydroxyacyl-CoA dehydrogenase) reversibly catalyses the third step of ß-oxidation, which takes place in the mitochondria and involves the FA catabolism. The results reflect a slight increase in the oxidative lipid metabolism for

TABLE 4 Effect of insectmeals in diets on the digestive enzyme activities of rainbow trout

Digestive enzymes (U·mg protein^{-1})	C	H1	H2	T1	T2	*SEM*
Acidic proteases	140.2	133.6	163.4	123.1	115.8	23.3
Alkaline proteases	83.2^{a}	112.4^{ab}	114.8^{ab}	153.6^{b}	144.8^{b}	10.5
Amylase	53.9^{b}	36.6^{ab}	29.2^{a}	34.8^{ab}	45.5^{ab}	5.18

Note: C: control diet (no insectmeal inclusion); H1 and H2: 150 and 300 g·kg^{-1} fishmeal replacement with Hermetia illucens, respectively; T1 and T2: 150 and 300 g·kg^{-1} fishmeal replacement with Tenebrio molitor respectively. $^{a, b}$ show statistically significant differences among diets ($p < .05$); Values are expressed as mean ± standard error of the mean (SEM; n = 4 tanks per diet, 2 fish per tank).

TABLE 5 Effect of insectmeals in diets on key enzymes in hepatic metabolism of rainbow trout

Enzymes (mU·mg protein^{-1})	C	H1	H2	T1	T2	*SEM*
β-hydroxyacyl-CoA dehydrogenase (HOAD)	19.2^{abc}	22.0^{bc}	17.7^{ab}	15.8^{a}	24.1^{c}	1.89
Pyruvate kinase (PK)	15.4	17.6	18.9	24.0	23.7	2.93
Citrate synthase (CS)	17.5^{ab}	16.9^{ab}	14.5^{a}	15.6^{ab}	19.7^{b}	1.62
Lactate dehydrogenase (LDH)	764.1	911.6	797.5	809.1	828.4	89.6
Fructose 1,6-biphosphatase (FBPase)	10.5	10.4	8.70	9.93	11.1	1.08
Glycerol kinase (GyK)	15.0	19.9	15.0	18.7	19.7	1.59
Glutamate dehydrogenase (GDH)	251.9	274.2	253.2	293.6	340.7	32.4
Glutamate pyruvate transaminase (GPT)	48.9	75.6	51.1	61.8	70.6	10.2
Glutamate oxaloacetate transaminase (GOT)	459.1^{ab}	423.0^{a}	417.9^{a}	461.0^{ab}	528.0^{b}	30.4

Note: C: control diet (no insectmeal inclusion); H1 and H2: 150 and 300 g·kg^{-1} fishmeal replacement with Hermetia illucens, respectively; T1 and T2: 150 and 300 g·kg^{-1} fishmeal replacement with Tenebrio molitor respectively. $^{a, b, c}$ show statistically significant differences among diets ($p < .05$); Values are expressed as mean ± standard error of the mean (SEM; n = 4 tanks per diet, 2 fish per tank).

H1 and T2, more marked for T2 (Table 5); that could cause a higher mobilisation of the fat, which could also be linked to the low VSI showed by these fish (Table 3). However, these differences were not statistically significant when comparing with C diet.

Two groups of results were observed when talking about glucose and amino acidic metabolisms (Table 5). The first one showed differences between diets with IM (CS and GOT). For CS, T2 had the highest activity, being statistically different from H2 ($p < .05$). For GOT, T2 also had the highest activity, being statistically different from H1 and H2. The greater activity of these two enzymes in T2 would suggest an increase of the protein catabolism to obtain energy, maybe derived from a different quality of the protein. However, this disruption on the intermediary metabolism of fish fed with T2 was not in direct consonance with a disruption on growth, probably due to a compensation derived from the greater digestibility of the protein observed in T2 (Table 3). The second group of results for hepatic enzymes on intermediary metabolism (PK, GyK, GDH and GPT) did not show any statistical differences (Table 5). It is easy to notice a synchronised overall increase on their activity when comparing IM results with those of control diet. As such, the present study encourages the possibility of increasing the amount of FM replacement with IM on further studies, in order to, maybe, find significant differences on their activity. These results are partially supported by those of Chemello (2020), since neither did they describe a significant increase for GDH or GPT after treatments with TM. The differences between their GOT results and those of the present study could be due to their diets having different sources of protein. In general, fish fed with T2 showed an increase on the oxidation of lipids, glucose and amino acids, and therefore a higher aerobic catabolism.

When the antioxidant capacity in liver was evaluated, fish fed with IM showed an increase on the antioxidant parameters SOD and CAT, and a decrease in lipid oxidative damage (MDA) respect to control diet (Table 6). Greater values in GR and GPX were observed on TM diets, but without statistical significance when comparing with control diet. Possibly, the increase on intermediary metabolism resulted in an increase of reactive oxygen species (ROS) production, and therefore a pro-oxidative cellular environment. However, the decrease on the MDA (Table 6) shows the efficacy of antioxidant mechanisms and the maintenance of redox homeostasis, without

TABLE 6 Effect of insectmeals in diets on antioxidant enzyme activities and levels of lipid peroxidation in liver of rainbow trout

Antioxidant enzymes and lipid peroxidation	C	H1	H2	T1	T2	*SEM*
Superoxide dismutase (SOD)	289.6^{a}	425.5^{b}	342.7^{ab}	396.6^{ab}	425.9^{b}	34.9
Catalase (CAT)	100.1^{ab}	123.9^{bc}	96.6^{a}	129.1^{cd}	152.5^{d}	8.24
Glutathione peroxidase (GPX)	26.1^{ab}	29.9^{ab}	24.6^{a}	27.5^{ab}	31.1^{b}	1.92
Glutathione reductase (GR)	10.8^{abc}	8.14^{ab}	8.0^{a}	12.5^{c}	12.2^{bc}	1.33
Glucose−6-phosphate dehydrogenase (G6PDH)	19.5	23.8	18.6	24.4	25.0	2.65
Malondialdehyde (MDA)	36.6^{b}	28.7^{a}	32.6^{ab}	32.4^{ab}	28.1^{a}	1.93

Note: C: control diet (no insectmeal inclusion); H1 and H2: 150 and 300 g·kg^{-1} fishmeal replacement with Hermetia illucens, respectively; T1 and T2: 150 and 300 g·kg^{-1} fishmeal replacement with Tenebrio molitor respectively. SOD and CAT expressed as U·mg protein^{-1}, GPX and GR as mU·mg protein^{-1}, and MDA as nmol·g tissue^{-1}. $^{a, b, c, d}$ show statistically significant differences among diets ($p < .05$); Values are expressed as mean ± standard error of the mean (SEM; n = 4 tanks per diet, 2 fish per tank).

damage derived from oxidative stress. This matches the results of Henry (2018a) on rainbow trout fed with TM; their study described an overall increase of SOD, CAT, GPX, GR and G6PDH, and a strong decrease in MDA. However, it would be interesting to point that they studied these enzymes in different parts of fish intestine (pyloric caeca, proximal and distal intestine), and that this relation with the present study was especially strong in proximal intestine. Taufek (2016) described also an increase in CAT for African catfish (*Clarias gariepinus*) liver after feeding cricket meal (*Gryllus bimaculatus*), while SOD and GST (glutathione S-transferase, not analysed) only suffered slight non-significant increases. Li (2017) described an increase in CAT for Jian carp (*Cyprinus carpio* var. Jian) serum after feeding black soldier fly, while SOD and MDA remained still among diets. Another work on yellow catfish fed with TM (Su, 2017) highlighted similar results, with an increase in plasma SOD activity both before and after an *Edwadsiella ictaluri* challenge, and a decrease in MDA activity prior to bacterial challenge. The general tendency of the bibliography is that the use of IM for fish increases the antioxidant defences of the animals while reducing the damage generated by lipid peroxidation. It has already been described that chitin and some of its derivatives can generate different antioxidant influences, such as a direct radical species scavenging effect and the increase of intracellular glutathione (Ngo & Kim, 2014). This, together with the previously mentioned results on intermediary metabolism, support the theory that T2 could have had a pro-oxidative effect on metabolism at the same time that it gave them tools to deal with it, causing no special oxidative damage.

Chitin can also have multiple effects on the immune system due to its polymeric structure (Lee, 2008), and its immunostimulant effect in fish has previously been described (Esteban, 2001; Gopalakannan & Arul, 2006; Kumar, 2019; Mousavi, 2020). Thus, different enzyme activities in plasma with a role in the innate immune response were analysed, such as lysozyme, esterase, anti-protease, acid phosphatase, alkaline phosphatase and peroxidase. In addition, the adaptive immune system was also evaluated by measuring the presence of total immunoglobulins (Ig) in plasma.

The lysozyme activity remained invariable between the different treatments (Table 7). Sankian (2018) described a linear increase of lysozyme activity with inclusion of TM in the diet of *Siniperca scherzeri*, getting to be significant at 300 g·kg^{-1} inclusion. This difference could be due to the fact that the IM inclusion level of the present study was lower, since the diet with the highest TM inclusion reached only 102.7 g·kg^{-1}. Nevertheless, the result matched Henry (2018b), who did not describe a significant difference in lysozyme activity when feeding TM meal to *Dicentrarchus labrax* at an even higher inclusion level of 247.5 g·kg^{-1}.

In the case of IM diets, an increase in anti-protease activity was observed, being significantly remarkable on H1 respect to control diet (Table 7). In contrast, the inclusion of IM in the diets showed a reduction in the presence of acid and alkaline phosphatase respect to control diet, being statistically significant only on H2, T1 and T2 for alkaline phosphatase ($p < .05$; Table 7). The acid and alkaline phosphatases have been showed as indicatives of tissue damage in other animals (Molina, 2005; Moreno, 2003). These results would also be in accordance with the maintenance of redox homeostasis previously described for fish fed with IM diets, since an increased oxidative stress environment can be related to tissue damage (Wang, 2019; Wu & Zhou, 2013).

In the case of peroxidase activity, there were no differences between treatments (Table 7). The part concerning TM matched the analysis of Sankian (2018) on serum myeloperoxidase after feeding TM to mandarin fish (*Siniperca scherzeri*). However, the present study, as well as that of Sankian (2018), were nutritional-focused studies with no pathogen challenges. It is possible that the exposure to a real pathogen aggression could have had the capacity to increase the expression of peroxidases or other immunological parameters.

Fish fed with IM showed a higher amount of total Ig, being statistically different for T1 (Table 7). In the case of HI diets, it seems there is a linear relation with the inclusion level in the diets. This result matches partially the works of Sangma and Kamilya (2015a, 2015b), where they described an increase in serum protein with different inclusion levels of chitin for *Catla catla* diet, as well as the work of

Immune parameters	C	H1	H2	T1	T2	SEM
Lysozyme activity	6.22	6.17	7.45	5.48	6.39	0.58
Esterase	378.6	365.6	310.5	330.2	371.6	19.2
Anti-protease activity	201.2^{a}	291.4^{b}	249.2^{ab}	248.5^{ab}	271.7^{ab}	16.8
Acid phosphatase	478.0	428.9	441.3	384.4	355.2	42.6
Alkaline phosphatase	1617.5^{b}	1,203.7^{ab}	1,152.9^{a}	1,088.4^{a}	1,075.6^{a}	98.0
Peroxidase	0.18	0.20	0.21	0.20	0.17	0.01
Total Ig	16.8^{a}	20.1^{ab}	26.9^{ab}	32.6^{b}	27.7^{ab}	2.93

Note: C: control diet (no insectmeal inclusion); H1 and H2: 150 and 300 g·kg^{-1} fishmeal replacement with Hermetia illucens, respectively; T1 and T2: 150 and 300 g·kg^{-1} fishmeal replacement with Tenebrio molitor respectively. Lysozyme activity expressed as μg · mL HEWL^{-1}(Hen Egg White Lysozyme), Esterase as mU·mg prot^{-1}, anti-protease activity as U anti-protease·mg prot^{-1}, acid and alkaline phosphatase as mU·mg protein^{-1}, peroxidase as optical density and total Ig as mg·mL^{-1}. $^{a, b, c}$ show statistically significant differences among diets ($p < .05$); Values are expressed as mean ± standard error of the mean (SEM; n = 4 tanks per diet, 2 fish per tank).

TABLE 7 Effect of insectmeals in diets on immune parameters in plasma of rainbow trout

TABLE 8 Effect of insectmeals in diets on proximal composition and fatty acid profile in fillets of rainbow trout

Proximate composition (g·kg^{-1} fish fillet on wet matter)	C	H1	H2	T1	T2	SEM
Protein	186.11	191.67	190.67	188.33	185.33	2.44
Fat	12.80	16.58	12.70	17.46	13.03	3.10
Moisture	761.1	761.7	754.6	757.8	755.7	1.99
Ash	13.69	13.63	13.58	13.29	13.80	0.13
Fatty acids in fillet (g·kg^{-1} fish fillet on wet matter)						
12:0 (*lauric*)	-	0.29^{a}	0.66^{b}	-	-	0.66
14:0 (*myristic*)	0.61	0.71	0.75	0.78	0.63	0.06
16:0 (*palmitic*)	3.51	3.45	3.36	3.91	3.14	0.30
16:1*n*−7 (*palmitoleic*)	0.76	0.77	0.80	0.99	0.79	0.08
18:0 (*stearic*)	0.85	0.79	0.77	0.89	0.72	0.08
18:1*n*−9 (*oleic*)	3.90^{a}	4.40^{ab}	4.66^{ab}	6.28^{b}	5.46^{ab}	0.45
18:1*n*−7 (*vaccenic*)	0.59	0.56	0.53	0.66	0.52	0.05
18:2*n*−6 (*linoleic*)	1.94^{a}	2.12^{a}	2.36^{ab}	3.18^{c}	3.00^{bc}	0.22
18:3*n*−3 (*linolenic*)	0.37^{a}	0.38^{a}	0.38^{a}	0.51^{b}	0.44^{ab}	0.04
18:4*n*−3 (*stearidonic*)	0.15	0.17	0.17	0.22	0.17	0.03
20:1*n*−9 (*eicosenoic*)	0.45^{a}	0.43^{a}	0.47^{a}	0.72^{b}	0.58^{ab}	0.07
20:4*n*−6 (*arachidonic*)	0.22^{b}	0.19^{ab}	0.17^{ab}	0.20^{ab}	0.14^{a}	0.02
20:4*n*−3 (*eicosatetraenoic*)	0.15	0.11	0.10	0.14	0.13	0.02
20:5*n*−3 (*ecosapentaenoic; EPA*)	1.25^{b}	1.08^{ab}	0.88^{a}	1.11^{ab}	0.81^{a}	0.11
22:1*n*−11 (*cetoleic*)	0.29^{a}	0.26^{a}	0.25^{a}	0.42^{b}	0.33^{ab}	0.03
22:5*n*−3 (*docosapentaenoic; DPA*)	0.43^{b}	0.33^{ab}	0.29^{a}	0.34^{ab}	0.28^{a}	0.04
22:6*n*−3 (*docosahexaenoic; DHA*)	4.74^{b}	3.81^{ab}	2.99^{ab}	3.16^{ab}	2.30^{a}	0.61
SFA (*saturated fatty acids*)	4.98	5.25	5.54	5.58	4.49	0.45
MUFA (*monounsaturated fatty acids*)	5.97^{a}	6.43^{a}	6.71^{a}	9.08^{b}	7.69^{ab}	0.66
PUFA (*polyunsaturated fatty acids*)	9.26	8.17	7.35	8.87	7.28	0.80
n−3	7.09^{b}	5.86^{ab}	4.81^{ab}	5.49^{ab}	4.14^{a}	0.74
n−6	2.17^{a}	2.31^{a}	2.53^{ab}	3.38^{c}	3.14^{bc}	0.22
n−6/*n*−3	0.33^{a}	0.40^{ab}	0.53^{bc}	0.61^{c}	0.76^{d}	0.05

Note: C: control diet (no insectmeal inclusion); H1 and H2: 150 and 300 g·kg^{-1} fishmeal replacement with Hermetia illucens, respectively; T1 and T2: 150 and 300 g·kg^{-1} fishmeal replacement with Tenebrio molitor respectively. $^{a, b, c, d}$ show statistically significant differences among diets ($p < .05$); n−6/n−3 is a non-dimensional ratio. Values are expressed as mean ± standard error of the mean (SEM; n = 4 tanks per diet, 2 fish per tank).

Kumar (2019), where similar parameters were measured after a parasite challenge and similar chitin inclusions.

Some of the differences in the data presented could be related to the differences in the chitin content between IMs (Table 1). Fish fed with H1 and T2 showed similarities in some variables, such as HOAD for intermediary metabolism, and SOD and MDA for antioxidant capacity, while those differences were not as obvious on H2 and T1. This could also be due to the fact that H1 and T2 had very similar amounts of chitin on their compositions (6–9 g·kg^{-1} feed, calculated from the IM chitin composition), whilst T1 had a lower concentration, and H2 could be reaching a theoretical toxicity barrier (Shiau & Yu, 1999), having double the amount of this compound in its composition.

Different sources of proteins and lipids may affect the fillet composition, as well as the quality of the final product. In this case the inclusion of IM, as source of both proteins and lipids, did not affect to the proximate composition of the fillet (Table 8). This coincides partially with the bibliography on rainbow trout fed IM, since most authors highlight little to none differences in fillet proximate composition after IM treatments, showing in some cases a reduction in fillet lipids and a compensatory increase in fillet moisture as IM is increased (Belforti, 2015; Dumas, 2018; Mancini, 2018; Sealey, 2011). This can also apply when reviewing the literature around whole-body proximate composition (Borgogno, 2017; Rema, 2019; St-Hilaire, 2007). However, the work of Renna (2017) observed an increment in the fat content of rainbow trout fillet after substituting up to 500 g·kg^{-1} FM in the diet with HI meal which, as the work itself states, could be due to the different rearing substrate of the insects used. Nevertheless, it is possible that the present study did not reach enough IM inclusion levels to match properly those same results.

The FA composition in fish fillet generally reflects the FA composition of the feeds (Turchini, 2009). The most remarkable differences in the FA profile of the feeds were their content of MUFA with a marked increase in TM diets, a decrease in EPA, DPA and DHA in all experimental diets, and an increase in omega-6 FA (*n-6*), specifically in linoleic acid. Another pronounced change would be the higher content in oleic acid in TM diets (Table 2). These FA variations have been reflected in the FA profiles of the fillets (Table 8). The most important changes were an increase of MUFA and *n-6* FA, together with a decrease in *n-3* as FM was replaced with IM, being especially high the increases on oleic FA for T1, the increase on *n-6* for TM diets, and the decreases on EPA, DPA and DHA for almost all experimental treatments. The health benefits of incorporating fish in the human diet for their content in *n-3* FA are well known; therefore, the lower content of these *n-3* FA in the fillet could involve a decrease in the perception of the fish quality by the consumers. Similar results have been found by other authors (Belforti, 2015; de Haro, 2016; St-Hilaire, 2007), which seems to be one main concern when dealing with IM based diets for fish. Thus, the search for strategies to improve this aspect will be critical for the implementation of the use of IM in commercial aquafeeds. One of the advantages on the use of insects is that they exhibit a high easiness on changing their composition according to the substrate used for their growth. Promising results have been obtained by Barroso (2017) and Danieli (2019); they proved the possibility of changing the proportion of PUFA in HI larvae composition by dietary modification. Hence, this consideration could be a major factor to have in mind in order to improve the *n-3* FA profile of fish whose diet is based on IM.

The study has covered diverse aspects related to growth efficiency, health and welfare status, and composition of the fillet, comparing two different IM at two different levels under the same experimental conditions. In general, the inclusion up to 109.1 g·kg^{-1}g/kg of HI and 102.7 g·kg^{-1}g/kg TM (300 g·kg^{-1}g/kg FM replacement) in diets for rainbow trout does not negatively affect the growth, protein utilization and the physiological status of the fish for the parameters evaluated. The replacement of FM with TM at 300 g·kg^{-1} showed a higher digestibility of the protein, a reduction of VSI, and a higher aerobic catabolism, generating a pro-oxidative environment that was compensated. Between IM diets, fish fed with H2 showed the most remarkable differences respect to T2 in intermediary metabolism and antioxidant activity, revealing the importance of the IM composition in its use for aquafeeds.

In contrast, the 300 g·kg^{-1} FM replacement level negatively affected the content of *n-3* FA in the fillet of fish fed with IM diets, which could lastly influence the perception of the consumers as an important source of *n-3* in a healthy diet.

Further studies are required to evaluate the effect of the use of feeds with IM for a longer time, and to determine the eligibility of the most appropriate IM to target different fish species, as well as to test different strategies to compensate the weakness of the IM inclusion, in order to maximize the potential of these protein sources.

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DATA AVAILABILITY STATEMENT

Research data are not shared.

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CHAPTER 4 – Study 2

animals

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Article

Fishmeal Dietary Replacement Up to 50%: A Comparative Study of Two Insect Meals for Rainbow Trout (*Oncorhynchus Mykiss*)

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Simple Summary: The reduction of dependence on fishmeal as a main protein source for aquafeeds remains a big problem in reaching sustainable aquaculture. Several alternatives to this ingredient are being tested and developed, insects being one of the most promising. The present study included two different insect species (black soldier fly, *Hermetia illucens*, and yellow mealworm, *Tenebrio molitor*) in the formulation of diets for rainbow trout (*Oncorhynchus mykiss*) against one typical fishmeal-based diet. Different parameters related to both the efficiency of these diets and their physiological repercussions were analysed. Yellow mealworm proved to be the best alternative for the growth and nutrition of rainbow trout, possibly due to some changes described in protein utilization and intestine histology, while other parameters revealed the possible usage of insect meals as functional ingredients due to their repercussions on preventing tissue damage.

Abstract: The demand of optimal protein for human consumption is growing. The Food and Agriculture Organization (FAO) has highlighted aquaculture as one of the most promising alternatives for this protein supply gap due to the high efficiency of fish growth. However, aquaculture has been facing its own sustainability problem, because its high demand for protein has been traditionally satisfied with the use of fishmeal (FM) as the main source. Some of the most promising and sustainable protein substitutes for FM come from insects. The present manuscript provides insight into an experiment carried out on rainbow trout (*Oncorhynchus mykiss*) with a 50% replacement of FM with different larvae insect meals: *Hermetia illucens* (HI), and *Tenebrio molitor* (TM). TM showed better results for growth, protein utilization and more active digestive function, supported by intestinal histological changes. Liver histology and intermediary metabolism did not show relevant changes between insect meals, while other parameters such as antioxidant enzyme activities and tissue damage indicators showed the potential of insect meals as functional ingredients.

Keywords: black soldier fly; mealworm; fishmeal replacement; rainbow trout; aquaculture; fish nutrition

1. Introduction

Albeit at a slower speed than some decades ago, the global population is expected to keep increasing and reach 8.5 billion in 2030, 9.7 billion in 2050, and 10.9 billion in 2100 [1]. As a consequence of this increment, the demand of adequate protein for human consumption is also increasing. Aquaculture is one of the most promising alternatives to satisfy this demand due to the high efficiency of fish growth [2], the rapid development of the aquaculture industry itself, and the adequate calories—protein ratio of fish [3]. However, because many of the fish cultivated for human consumption require high protein levels to grow appropriately, aquaculture has been facing its own sustainability problems in the last few decades. These protein requirements have been traditionally satisfied with the use of fishmeal (FM) from wild-caught fish and as a by-product of extractive fishing practices [4]. Due to the fast growth of aquaculture, these ingredients are considered as non-sustainable in the long term.

Many efforts have been carried out from both research and aquaculture industries to partially replace FM with sustainable ingredients in fish feeds, without impairing fish growth and while giving insight into these sustainable ingredients. Alternatives such as vegetable ingredients [5,6], yeast [7,8], or microalgae [9,10] are some of the ingredients that are being studied currently. Following this line, the present study is focused on insects as one of the most interesting protein substitutes for FM [11–14]. Setting aside the interspecific differences, as well as the harvesting time of larvae [15], the amino acidic proportions of the most typically studied insects tend to match that of FM [16,17]. Insects also reproduce and grow easily, have very efficient growth ratios, and require low amounts of space and energy to be produced [18]. Hence, their potential as a good source of sustainable animal protein is promising. Because the Food and Agriculture Organization (FAO) has mentioned zero hunger, sustainable communities, and life below water as three of its 17 Sustainable Development Goals of the 2030 Agenda [19], it is easy to assume that both aquaculture and insect production might consequentially play important roles in the upcoming years or decades.

Several manuscripts have proven the efficiency of insect meals (IMs) in different fish species [20–24], revealing the importance of both the insect and the fish species involved. For salmonids, the inclusion of IMs in feeds is, in general, well accepted. As an example of unaffected growth, Terova [25] replaced up to 30% FM with *Hermetia illucens* (HI) in rainbow trout feed (*Oncorhynchus mykiss*). In the case of Atlantic salmon (*Salmo salar*), it was already proven that FM could be replaced entirely, using HI as one of the chosen protein sources [26]. Another experience [27] tested two hydrolysed IMs (yellow mealworm, *Tenebrio molitor* (TM) and superworm, *Zophobas morio*) on fingerlings of sea trout (*Salmo trutta* m. *trutta*) at 40% FM replacement and noticed almost no changes in growth or protein use. Moreover, another experiment [28] did not note changes in growth for rainbow trout, substituting FM completely with TM. These and other published data support the idea that, with the due differences among species, a partial replacement of FM with IM has no adverse effects on the growth of most fish. Moreover, functional properties such as a possible enhancement of both the immunological and the antioxidant systems have been attributed to IMs, possibly due to compounds such as chitin or its derivatives [14,21,29–33].

The European Commission approved the use of seven insects as ingredients in aquafeeds [34]. Due to their relative availability, HI and TM are two of the most broadly studied insects for animal nutrition. Thus, the IM industry has a big potential and requires research studies to validate the use of IMs as an alternative ingredient in feed for aquaculture.

Following the results of a previous study with 15–30% FM replacement (5–10% IM inclusion level) [32], but increasing the FM replacement to 50% (18% IM inclusion level) in feed for rainbow trout, the present manuscript provides insights on the effects of two different IMs for several aspects, from growth to final composition of the fillets, while evaluating the physiological status of the fish and their possible consequences on health and welfare status.

2. Materials and Methods

2.1. Experimental Diets

Whole dried insects from two different species in larval stage, *Hermetia illucens* (HI; Entomotech S.L., Almería, Spain) and *Tenebrio molitor* (TM; Mealfood Europe S.L., Salamanca, Spain) were used for this study, processed as insect meals (IMs). IMs were analysed before the formulation of the diets (Table 1). A total of three isoproteic (43.3%) and isolipidic (17.4%) diets were formulated (Table 2): a control diet with no IM (C), and two diets with 18% diet inclusion (50% fishmeal replacement) of the cited IMs: H18 (HI), and T18 (TM). Ingredients were provided by 'Lorca Nutrición Animal S.A.' (Murcia, Spain). Methionine and lysine were added to diets to meet the nutritional requirements of rainbow trout [35,36], manufactured by LifeBIOENCAPSULATION S.L. (Almería, Spain), and extruded as pellets of 3 mm. The dough was passed through a single screw laboratory extruder (Miltenz 51SP, JSConwell Ltd., Palmerston North, New Zealand). The extruder barrel had four sections, with a temperature per section of (from inlet to outlet) 100 °C, 95 °C, 90 °C and 85 °C, respectively. Pellets were kept in a drying chamber at 30 °C for 24 h (Airfrio, Almería, Spain) and stored in sealed plastic bags at −20 °C until they were used.

Table 1. Proximate and amino acids compositions of insect meals (IMs).

Proximate Composition	*Hermetia illucens* (HI)	*Tenebrio molitor* (TM)
Crude protein (%)	28.5	39.1
Crude fat (%)	25.6	27.0
Crude ash (%)	9.75	3.42
Moisture (%)	8.00	5.00
Calcium (g/Kg)	35.2	0.93
Phosphorus (g/Kg)	7.00	7.50
Calcium–phosphorus ratio	5.03	0.12
Chitin (%)	7.50	5.90
Amino acid composition (g/100 g IM)		
Asp (aspartate)	2.92	3.71
Thr (threonine)	0.95	1.44
Ser (serine)	1.43	2.49
Glu (glutamate)	3.19	4.98
Pro (proline)	1.58	3.04
Gly (glycine)	1.84	2.87
Ala (alanine)	2.37	3.92
Cys (cysteine)	0.13	0.24
Val (valine)	1.42	2.32
Met (methionine)	0.47	0.57
Ile (isoleucine)	0.91	1.31
Leu (leucine)	1.86	2.96
Tyr (tyrosine)	2.23	4.47
Phe (phenylalanine)	2.16	3.07
His (histidine)	1.07	1.77
Lys (lysine)	1.94	2.49
Arg (arginine)	1.24	1.81

Table 2. Formulation, proximate, and amino acid composition of experimental diets.

Ingredients (%; on Wet Basis)	C	H18	T18
Fishmeal	35.9	18.0	18.0
HI meal	0.00	18.0	0.00
TM meal	0.00	0.00	18.0
Wheat gluten	10.5	15.4	11.9
Soy protein concentrate	15.5	18.3	17.0
Wheat meal	16.4	11.5	17.0
Soy lecithin	1.30	0.50	0.50
Fish oil	12.2	9.50	9.00
Vitamins and minerals	2.00	2.00	2.00
Goma guar	2.00	2.00	2.00
Blood meal	4.00	4.00	4.00
Methionine	0.20	0.50	0.50
Lysine	0.00	0.40	0.10
Total	100	100	100
Proximate composition (%; on wet basis)			
Moisture	7.43	7.74	7.21
Total crude protein	43.9	42.8	43.1
Total crude fat	17.2	17.1	17.9
Ash	7.41	6.45	6.11
Calcium	0.51	0.26	0.45
Phosphorus	0.33	0.24	0.24
Calcium–phosphorus ratio	1.6	1.1	1.9
Amino acid composition * (g/100 g feed)			
Asp (aspartate)	2.89	1.86	2.29
Thr (threonine)	1.65	1.25	1.38
Ser (serine)	1.59	1.22	1.46
Glu (glutamate)	6.55	5.96	5.96
Pro (proline)	2.15	2.11	1.84
Gly (glycine)	1.78	1.16	1.53
Ala (alanine)	1.73	1.05	1.58
Cys (cysteine)	0.68	0.67	0.64
Val (valine)	2.07	1.65	1.87
Met (methionine)	1.22	1.13	1.26
Ile (isoleucine)	1.93	1.55	1.63
Leu (leucine)	3.15	2.56	2.79
Tyr (tyrosine)	1.11	0.87	1.42
Phe (phenylalanine)	1.89	1.63	1.93
His (histidine)	1.03	0.84	1.03
Lys (lysine)	2.66	1.88	2.18
Arg (arginine)	2.50	1.86	2.04

Vitamin and mineral premix (% unless otherwise specified): vitamin A 2,000,000 UI; vitamin D3: 200,000 UI; vitamin E: 1.2; vitamin K3: 0.26; vitamin B1: 0.3; vitamin B2: 0.3; vitamin B6: 0.2; vitamin B9: 0.15; vitamin B12: 0.001; vitamin H: 0.03; inositol: 5; betaine: 5; calcium pantothenate: 1; nicotic acid: 2; Co: 0.006; Cu: 0.09; Fe: 0.06; I: 0.005; Mn: 0.095; Se: 0.0001; Zn: 0.075; Ca: 19; K: 2.4; Na: 4.1. C: control diet (no IM inclusion); H18: 18% *Hermetia illucens* inclusion (HI); T18: 18% *Tenebrio molitor* inclusion (TM). * Calculated from basic amino acids of ingredients.

2.2. Experimental Animals and Rearing Conditions

A total of 360 female rainbow trout with an initial weight of 14.6 ± 0.2 g from a commercial farm (Piscifactoría Fuente del Campillo, Guadalajara, Spain) were transported to the experimental facilities of the Aquaculture Research Centre of "Instituto Tecnológico Agrario de Castilla y León" (ITACyL). Fish stayed in acclimation for 15 days before the beginning of the growth trial, and then they were randomly allocated into 12 cylindrical fiberglass tanks (four replicas per treatment; 500 L) of a recirculating system, in groups of 30 animals. Once a day (9 a.m.), fish were fed by hand until apparent satiation was reached (maximum of 3% daily feed intake). During the growth trial (77 days), water temperature

(12.5 ± 1 °C), water dissolved oxygen (9.2 ± 1 mg/L), and room photoperiod (12 h light: 12 h dark) were monitored. Water ammonia and nitrite levels were analysed daily, and kept at optimal levels (ammonia < 0.1 mg/L and nitrite < 0.1 mg/L). The care and handling of rainbow trout were conducted according to specific regulations: The Directive of the European Union Council (2010/63/EU) [37] and the Spanish Government (Real Decreto 53/2013) [38]. The experiment was approved previously by the Bioethical Committee of "ITACyL" (Authorization number: 2017/19/CEEA).

2.3. Growth Trial and Samples Collection

Mortality and feed intake were monitored on a daily basis. Fish were measured and weighed every 21 days through a simple biometry procedure with a graduated ictiometer (±0.1 mm) and scale (±0.1 g), being previously fasted for one day and anesthetized with tricaine methanesulfonate (MS-222; 180 mg/mL). In order to take samples of the different tissues, the fish were sacrificed by an overdose of MS-222 (300 mg/mL).

Before the feeding trial, eight fish were randomly sacrificed to analyse the initial value of the protein in the fillet.

During the final two weeks of the experiment, faeces were gathered every 24 h in a settling column using a modified Guelph method [39], and frozen at −80 °C until they were analysed. At the end of the experiment, eight fish per diet (2 fish per tank) were randomly sampled and sacrificed. According to time sequence, the following were collected to be analysed individually: skin mucus, blood, liver, stomach, intestine with pyloric caeca, and fillet samples. Skin mucus samples were collected by scraping the dorso-lateral surface of the fish skin from cranial to caudal according to de Mercado et al. [40] and frozen at − 80 °C until processing. Blood samples were collected with heparinized syringes and their plasma was separated by centrifugation at 3500× *g* and 4 °C, for 15 min. Individual plasma samples were frozen at −80 °C until their analysis.

For enzyme determinations, samples were frozen in liquid nitrogen and kept at −80 °C until they were analysed. For tumour necrosis factor-alpha determination (TNF-α), distal intestine samples were kept in Allprotect Tissue Reagent (QiaGEn) and stored at −20 °C until protein extraction. The samples for histomorphology analyses were fixed in 4% buffered formalin for 48 h before dehydration and processing. For chemical analyses, the samples were directly frozen at −80 °C.

2.4. Histomorphology

2.4.1. Samples Processing

The fixed samples were dehydrated in increasing ethanol solutions (25%, 50%, 75%, and 100%) and embedded in synthetic paraffin. Histological sections (3–4 µm) were obtained by a rotary microtome (FINESSE ME+ Thermo Scientific©, Waltham, MA, USA), stained by hematoxylin and eosin technique for histomorphology studies and observed with light microscopy. All of the evaluations were performed by graded objective lens in five random regions for each stained tissue section with an Olympus EP50 microscope camera and an Olympus CX31 microscope.

2.4.2. Distal Intestine and Pyloric Caeca Histomorphology Analyses

Quantitative studies included the measurement of heights of villi and enterocytes, as well as widths of villi, *stratum compactum*, muscular layers (longitudinal and circular), and lamina propria as mean of three measures (apical, intermediate, and basal). The level of inflammatory infiltration in lamina propria, the level of loss of supranuclear vacuolization of enterocytes, and the relative position of enterocyte nuclei were measured through a subjective analysis.

2.4.3. Liver Histomorphology Analysis

Hepatocyte cytoplasm and hepatocyte nucleus measures were taken as quantitative variables. A qualitative analysis concerning the search of inflammatory patterns (necrosis and inflammation focuses) and hepatocyte intranuclear vacuolization was also carried out.

2.5. Analytical Determinations

For intermediary metabolism and antioxidant status, liver samples were individually homogenized in nine volumes of ice-cold 100 mM Tris-HCl buffer, containing 0.1 mM EDTA and 1 g/kg (v/v) Triton X-100, pH 7.8. This was followed by centrifugation at 30,000 *g* for 30 min, at 4 °C. For further enzyme assays, the supernatants were stored at −80 °C as aliquots.

The concentration of soluble protein in samples was determined by Bradford method [41], employing bovine serum albumin as a standard.

2.5.1. Chemical Analyses

AOAC methods [42] were used to analyse fat content and moisture of IMs, diets, and fish fillets. Protein content was determined with the Dumas method [43], using a nitrogen analyser (FP 528, LECO, St. Joseph, MO, USA), and with a conversion factor of 4.67 for HI, 4.75 for TM [44], and 6.25 for feeds and faeces. Acid-insoluble ash was used as marker in feeds and faeces to determine the apparent digestibility of the protein [45]. Phosphorus (P) was determined by molecular absorption spectrophotometry according to ISO standard [46], with a spectrophotometer (UV/Vis UV2, UNICAM, Cambridge, UK). Calcium was determined as described by Pessoa [47], with X-ray fluorescence method of Dispersive Energy. The method described by Gamage and Shahidi [48] was used to isolate chitin from IM, which was washed with acetone, dried, and weighed afterwards. For amino acids, samples of HI and TM were hydrolysed with 6 N HCl for 22 h at 110 °C [16]. The determination was performed by ion-exchange liquid chromatography and postcolumn continuous reaction with ninhydrin (Biochrom 30; Cambridge, UK). Tryptophan was not determined.

2.5.2. Digestive Enzymes Determination

Intestine with pyloric caeca and stomach were processed separately to determine digestive enzymes. Samples were first individually homogenized at 4 °C with distilled water (250 mg/mL). Acid protease activity was determined from stomach extracts, while amylase and alkaline protease activities were determined from the intestine and pyloric caeca extracts. The activity of amylase was determined through the Somogy–Nelson method [49], with soluble starch 20 g/kg as substrate, defining one unit of activity as the quantity of enzyme able to produce 1 µg of maltose per minute and mg of protein. Walter method [50] was used to measure the activity of alkaline protease, employing casein 10 g/kg as substrate. Anson method [51] was used to measure the activity of acid protease activity, with hemoglobin 5 g/kg as substrate. For both proteases, one unit of activity was defined as 1 µg of tyrosine produced per minute and mg of protein. The standard temperature for all digestive enzyme analyses was 37 °C.

2.5.3. Liver Intermediary Metabolism

The method described by Furné [52] was used to determine the enzymatic activity of fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11), pyruvate kinase (PK, EC 2.7.1.40), glutamate pyruvate transaminase (GPT; EC 2.6.1.2), glutamate oxaloacetate transaminase (GOT; EC 2.6.1.1), and glutamate dehydrogenase (GDH; EC 1.4.1.2). Enzymes were analysed at 25 °C, and changes in absorbance were monitored with a PowerWaveX microplate scanning spectrophotometer (BioTek Instruments, Winooski, VT, USA) to determine the enzyme activity.

2.5.4. Non-Specific Immune Status

Plasma Immunological Determinations

Lysozyme activity was performed using a turbidometric method [53] with *Micrococcus lysodeikticus* (Sigma, St. Louis, MO, USA). After reaction for 20 min at 35 °C, the absorbance was measured at 450 nm. A standard curve with hen egg-white lysozyme was used.

Total esterase activity was assayed according to Mashiter and Morgan [54] at 25 °C. P-nitrophenyl acetate (0.8 mM) was used as a substrate, and 1.6 mM acetazolamide as an inhibitor of carbonic anhydrase activity. The absorbance increase was measured at 405 nm for 5 min, after incubation for 10 min.

Anti-protease was measured according to Thompson [55]. The production of 4-nitroaniline was determined by the variation of the OD (optical density) at 410 nm for 30 min. Trypsin activity in absence of plasma was used as control (CAS 90002–07–7, Acofarma, Spain).

Phosphatase activity was determined according to Huang [56]. P-nitrophenyl phosphate (Sigma) was used as a substrate, a buffer at pH 10 ($NaHCO_3$/NaOH 0.05 M, $MgCl_2$ 1 mM) was used for alkaline phosphatase activity, and a buffer at pH 5 (CH_3COOH/$CH_3COOHNa$ 0.1 M, $MgCl_2$ 1 mM) to measure acid phosphatase activity. The measurement was performed at 405 nm for 30 min, at 37 °C.

Peroxidase activity was determined according to Mohanty and Sahoo [57]. A solution of 20 mM TMB (3, 3′, 5, 5′-Tetramethylbenzidine) was used as substrate. The samples were read at 450 nm after blocking reaction for 2 min. Plasma-free standard samples were measured as controls. The activity was expressed in OD (optical density).

Total immunoglobulin was determined according to Panigrahi [58]. After precipitation with polyethylene glycol, the immunoglobulins were separated from the total proteins. Total immunoglobulin content was calculated by subtracting the protein content resulting from the total protein content in the untreated plasma.

TNF-α Detection in Distal Intestine and Skin Mucus

For protein extraction, samples of the distal intestine and skin mucus were homogenized using beads and an ice-cold lysis buffer (Tris 20 mM, NaCL 100 mM, Triton X-100 0.05%, EDTA 5 mM, protease inhibitor cocktail 1X), in a bead mill homogenizer (Qiagen RETSCH tissuelyser). The homogenate was centrifuged for 25 min, at 12,000× *g* and 4 °C. The soluble proteins contained in the supernatant were stored at −20 °C until use. The cytokine TNF-α was determined following the indirect ELISA method described by Morales-Lange [59], with slight modifications according to Weththasinghe [33]. Briefly, 100 µL of sample diluted to 45 ng/µL in a carbonate buffer (60 mM $NaHCO_3$, pH 9.6) were seeded into 96-well plates (NUNC MAXISORPTM, Invitrogen), and incubated overnight at 4 °C. After blocking (5% Blotting-Grade Block, BioRad, Hercules, CA, USA; 2 h at 37 °C), plates were incubated for 90 min at 37 °C with 50 µL of the primary antibody (rabbit anti-TNFα, diluted 1: 200). Next, 50 µL of the secondary antibody (mouse anti-rabbit IgG-HRP, diluted 1: 7000) were added and incubated for 60 min at 37 °C. Finally, 100 µL of chromagen substrate 3,3′,5,5′- tetramethylbenzidine single solution (TMB, Thermofisher, Waltham, MA, USA) was added and incubated for 30 min at room temperature. The reaction was stopped with 50 mL of 1 N sulfuric acid and read at 450 nm on a Spectra Max microplate reader (Spectra Max M2; Molecular Devices, San José, CA, USA). The calibration curve was performed using serial dilutions of the corresponding epitope peptide ranging from 0 µg/mL to 1.2 µg/mL.

2.5.5. Liver Antioxidant Status and Fish Welfare Indicators

The procedure described by Pérez-Jiménez [60] was followed to determine superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), and glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49). The enzyme analyses were carried out at 25 °C, and the enzyme activity was determined using a PowerWaveX microplate scanning spectrophotometer (BioTek

Instruments, Winooski, VT, USA), through the monitorization of absorbance changes. Preliminary assays allowed the establishment of the optimal substrate and protein concentrations to measure the maximal activity of each enzyme. The millimolar extinction coefficients used for NADH/NADPH, DTNB, and H_2O_2 were 6.22, 13.6, and 0.039/mM·cm, respectively. The enzyme needed to inhibit half of the ferricytochrome C reduction rate was defined as one unit of SOD activity. For other enzymes, the amount of enzyme required to transform 1 µmol of substrate per minute was defined as one unit of enzyme activity. Malondialdehyde (MDA) level was used to quantify lipid peroxidation. MDA reacts in the presence of thiobarbituric acid to produce coloured thiobarbituric acid reacting substances (TBARS).

Plasma glucose and lactate levels were analysed with commercial colorimetric kits, following the instructions of the manufacturer (Glucose-TR, ref. 41011, Spinreact; Lactate, ref. 1001330, Spinreact). The absorbance was measured using a microplate reader (ELx800TM; BioTek Instruments, Inc., Winooski, VT, USA), in 96-well microplates.

2.6. Statistical Analysis

The software used for the statistical analyses was SAS system version 9.0 (SAS Institute Inc., Cary, NC, USA). A general linear model (PROC GLM) analysis of variance (ANOVA) was used to process the data, and the comparison of the means was performed by a Tukey test. Differences were considered significant when the p-value was <0.05. Values are showed as mean ± standard error of the mean.

3. Results and Discussion

3.1. Growth Performance

In general, the performance of all diets was within normal values, with an efficient FCR (Table 3). Fish fed with T18 showed the best overall growth performance with very similar values to C while H18 showed lower numbers for growth, being statistically different from T18, or even to C when talking about SGR, FCR, and the apparent digestibility coefficient of the protein (ADC_{prot}). Even though there are small discrepancies in the literature about the performance of HI as an ingredient for fish [61–64], the present results seem to follow the general conclusions of other trials in rainbow trout. Rainbow trout seem to have a higher tolerance to the inclusion of TM in diets [65,66] than that of HI [61,67], which could be due to the different levels of chitin in the composition of the insects, or their amino acid profiles. Chitin might have a positive influence over fish physiology as a functional ingredient [68], but the presence of this molecule tends to lower the digestibility of crude protein [69,70]. Because HI has higher levels of chitin in its body composition than TM (Table 1), this, together with results in other experiences [61,65,67,71] suggest that a 15–18% inclusion of HI in rainbow trout feed is a possible maximum level of inclusion for this species, while an 18% of TM or even more, is still compatible with optimal growth performance. In addition, the digestibility of the protein was higher in TM than HI; although the amino acid profile between IMs differed, the diets were supplemented with methionine and lysine to cover the nutritional requirements. This should had led to a similar growth between insect-based treatments, but the higher growth of T18 over H18 means that its higher digestibility played an important role, and consequently led to a higher growth than HI.

Table 3. Growth performance, protein utilization, and biometric indices of rainbow trout fed experimental diets.

Growth Performance	C	H18	T18	SEM	*p*-Value
IBW (g)	14.3	14.8	14.7	0.22	0.325
IBL (cm)	11.1	11.2	11.2	0.05	0.377
FBW (g)	76.4 ^{a,b}	69.4 ^{b}	81.9 ^{a}	2.41	0.016
FBL (cm)	18.0 ^{a,b}	17.6 ^{b}	18.5 ^{a}	0.16	0.010
SGR (%/day)	2.17 ^{a}	2.00 ^{b}	2.23 ^{a}	0.04	0.011
DFI (g/100 g fish·day)	1.57	1.62	1.57	0.02	0.267
FCR	0.90 ^{a}	0.98 ^{b}	0.88 ^{a}	0.02	0.006
Protein utilization					
PER	2.49 ^{a,b}	2.34 ^{b}	2.58 ^{a}	0.05	0.015
PPV (%)	49.2	48.6	51.8	1.23	0.196
ADC_{prot} (%)	92.6 ^{a}	81.0 ^{b}	91.2 ^{a}	1.05	<0.0001
Biometric indices					
CF (g/cm^3)	1.31	1.28	1.3	0.01	0.461
HSI (%)	1.26	1.44	1.29	0.07	0.244
VSI (%)	14.3	15.9	14.5	0.42	0.051

C: control diet (no IM inclusion); H18: 18% HI inclusion; T18: 18% TM inclusion. IBW: initial body weight; IBL: initial body length; FBW: final body weight; FBL: final body length; SGR (specific growth rate) = ((ln FBW-ln IBW)/days) · 100; DFI (daily feed intake) = (daily feed consumption (g)/biomass (g) at time) · 100; FCR (feed conversion ratio) = (total feed intake (g)/(FBW-IBW)); PER (protein efficiency ratio) = (total weight gain (g)/protein intake (g)); PPV (productive protein value) = ((protein gain (g)/protein intake (g)) 100); ADC_{prot} (apparent digestibility coefficient of the protein) = 100 – ((marker in diet (g)/marker in faeces(g)) (% protein in faeces/% protein in diet)·100); CF (condition factor) = (weight (g)/length^3 (cm)) 100; HSI (hepatosomatic index) = (wet liver weight/FBW)·100; VSI (viscerosomatic index) = (wet visceral weight/FBW) 100. $^{a, b}$ Show statistically significant differences among diets ($p < 0.05$); Values are expressed as mean ± standard error of the mean (SEM; $n = 4$ tanks per diet).

The mortality during the trial was around 3% without remarkable differences between diets (data not shown).

3.2. Histomorphology

3.2.1. Distal Intestine and Pyloric Caeca

On distal intestine (Figure **1**), no significant differences were described for villi, *stratum compactum*, longitudinal muscular layer, or lamina propria widths. Villi height was higher for T18 fish than H18 ($p < 0.05$), with an intermediate value for C fish. Enterocyte height was higher in T18 than in C ($p < 0.05$), with no significant differences between H18 and T18. The circular muscular layer was wider on C than on H18, while the total muscular layer was wider on C than on both IM treatments ($p < 0.05$). For qualitative analyses (Figure 2), slightly higher levels of both inflammatory infiltration and loss of intracellular vacuolization were highlighted for C diet. The different degrees of supranuclear vacuolization did not affect the enterocyte structure because most nuclei were positioned on the basal part of the enterocytes, showing no differences between treatments.

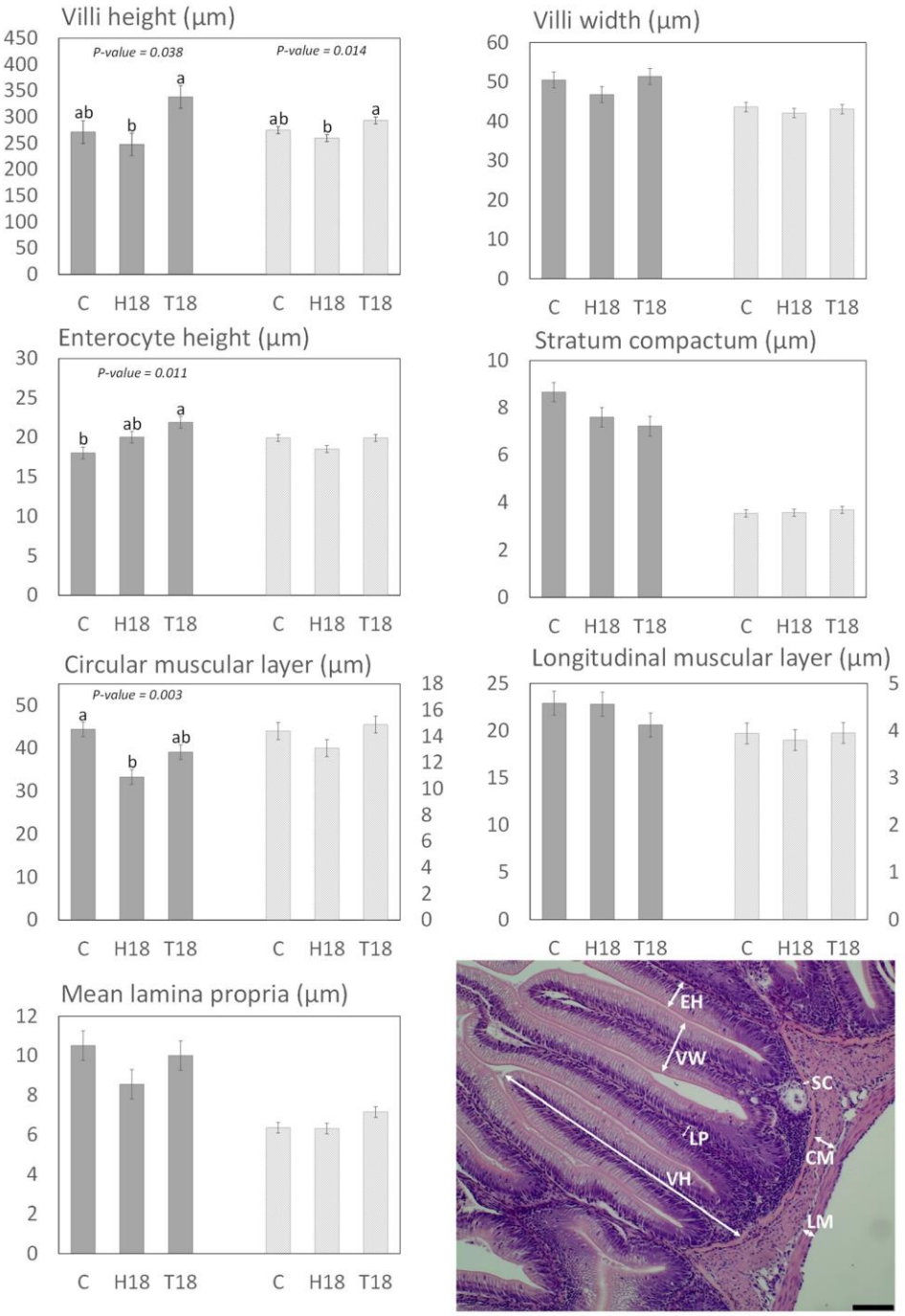

Figure 1. Histomorphology of rainbow trout gut. C: control diet; H18: 18 % HI inclusion; T18: 18 % TM inclusion. Grey bars: distal intestine measures; striped bars: pyloric caeca measures. a, b Show statistically significant differences among diets ($p < 0.05$); Values expressed as mean ± standard error of the mean (SEM; $n = 4$ tanks per diet, 2 fish per tank). Microphotograph representative of measures for gut: villi height (VH), villi width (VW), enterocyte height (EH), *stratum compactum* (SC), circular muscular layer width (CM), longitudinal muscular layer width (LM), lamina propria width (LP). Scale bar = 100 µm.

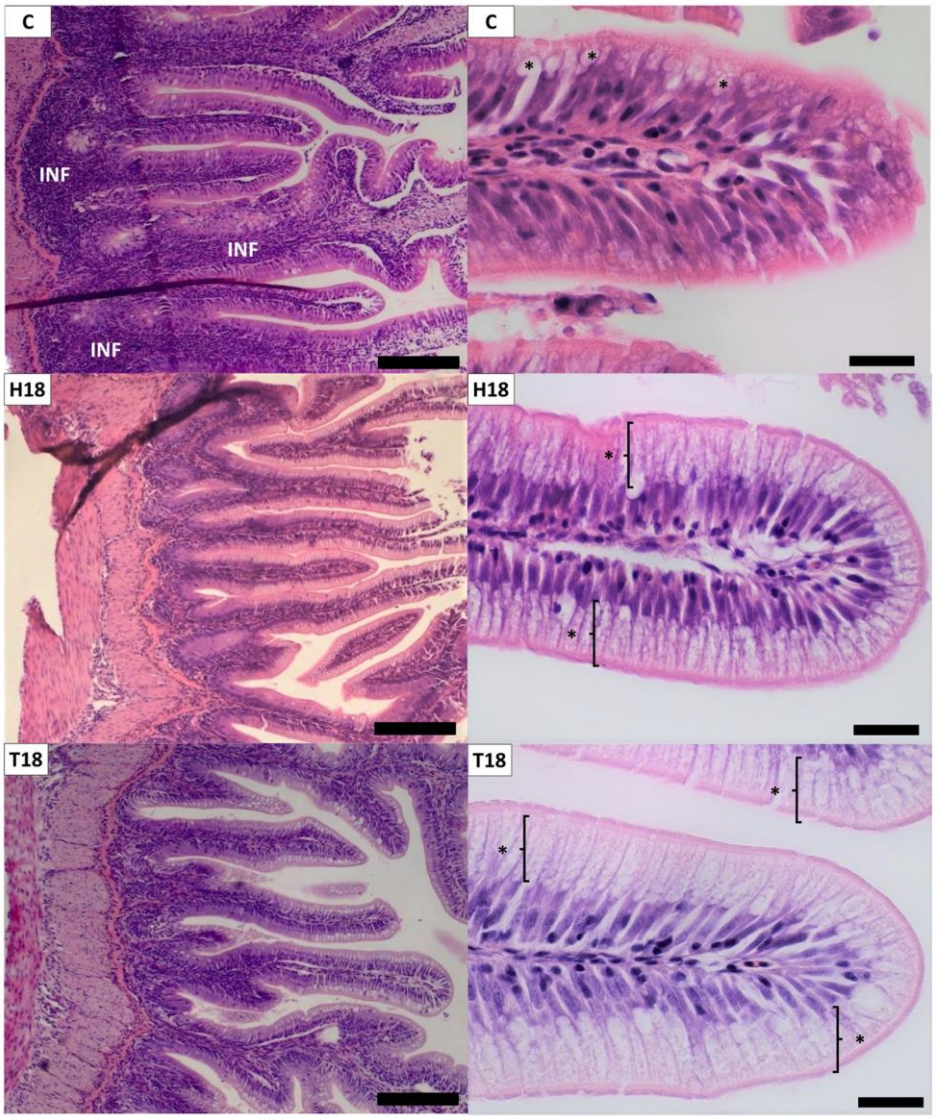

Figure 2. Histomorphology of rainbow trout distal intestine. C: control diet (no IM inclusion); H18: 18% HI inclusion; T18: 18% TM inclusion. Microphotographs of qualitative analyses: left photographs represent a comparative view of inflammatory infiltration (INF), pictures taken at 40× magnification, scale bars = 100 µm; right photographs represent a comparative view of vacuoles (marked with asterisks) in enterocytes, pictures taken at 400× magnification. Scale bars = 10 µm.

Few significant differences were found in pyloric caeca compared to the distal intestine. T18 showed the highest values for villi height (compared to H18; $p < 0.05$), while the rest of the results remained stable (Figure 1). For the qualitative analyses, no differences were observed for inflammatory infiltration or intracellular vacuolization, and most nuclei were described on the intermediate part with no differences between treatments.

The histology of insect-fed fish intestine has been extensively studied in the last few years [27,33,63,72–74]. However, due to the large number of variables involved in the studies, such as the fish species, the insect used to elaborate the feeds, the chosen intestine sections, or the analysed parameters, there is still work to be conducted. Villi height is one of the most frequently analysed parameters, being an indicator usually associated with gut health and growth performance. In this way, the results of this study match partially those of the current literature, because it has been described that similar inclusions of HI in fish feed tend to decrease villi height [67,71,75–77]. There are other cases in which no changes or even an increase in villi height was described with the addition of HI meal [63,78,79].

The case of TM is less studied and it seems that, with one exception [63], most studies describe no changes in villi height when dealing with this ingredient [27,73,80].

On pyloric caeca, the results were similar. However, this is a less studied variable for insect-fed fish, and to our knowledge, only two studies evaluated this parameter after a growth trial with HI treatments; one that matched the data of this study [77], and one that offered opposite results [81]. Considering that digestive efficiency and growth are directly related to gut anatomy alterations such as a decrease in the absorption surface [82–84], it is no surprise that the results of the present manuscript agree with the lower growth performance and protein digestibility described for HI, as well as the higher results on TM.

The circular muscular layer was also affected in the present study. The main finding was a decrease in the width for H18 with significant difference with respect to C diet ($p < 0.05$). Because contraction and relaxation of circular and longitudinal muscular layers lead to peristaltic movements, a different width of the circular muscular layer could alter the movement of the feed along the gut, affecting the intestinal bacterial growth [85] and ultimately the digestibility of the nutrients, consistent with the lower digestibility of the protein observed in fish fed with H18. Similar results were showed by Lu [76], while other authors have not described changes in muscular layer width [27,79,81]; there is even a case in which different insect species gave different results [63] with the inclusion of IMs. This divergence of data is probably caused by the different species of fish used.

It is interesting to notice that the differences in the degree of enterocyte supranuclear vacuoles loss (fewer vacuoles in C) are in consonance with the results on enterocyte height (lower in C, with a significant difference between C and T18). The presence or absence of lipidic vacuoles in enterocytes has been related to their height for other ectothermic species [86,87]. In this way, the different nature of the fat between diets (insect fat in H18 and T18), and their absorption process may have played an important role in the degree of supranuclear lipidic vacuoles in enterocytes. Furthermore, the work of Kumar [88] described how IMs could cause a protective effect against the problems of soybean meal [89] in salmonids. Considering that C diet also showed a slightly higher submucosa inflammatory infiltration, the lower degree of vacuolization and the consequential lower enterocyte height with respect to IM diets could be due to the lack of this protective effect. The three diets had a relatively high amount of vegetable ingredients, but even though H18 and T18 had the highest amounts of these ingredients, they showed the lowest levels of inflammatory signs.

3.2.2. Liver

No significant differences were found in any of the measured variables: hepatocyte nucleus and cytoplasm diameters, inflammatory patterns, or level of hepatocyte vacuolization (Figure 3). Liver histology has also been extensively studied for insect-fed fish [71,72,74,75,81]. One of the most frequent findings in several fish species, including rainbow trout, is that an increasing proportion of IMs in the feed tends to increase the number of lipidic vacuoles in hepatocytes, while other related variables are mostly unaffected [71,78,81]. This discrepancy with our results could be due to the different size and feeding period of the fish involved, because the cited study in rainbow trout was performed with bigger fish. The work of Kumar [88], however, had similar conclusions to those of the present study.

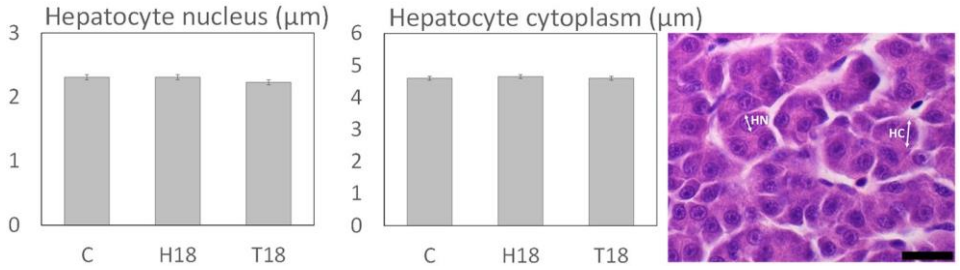

Figure 3. Histomorphology of rainbow trout liver. C: control diet (no IM inclusion); H18: 18 % HI inclusion; T18: 18 % TM inclusion. Values are expressed as mean ± standard error of the mean (SEM; n = 4 tanks per diet, 2 fish per tank). Microphotograph representative of measures for liver: hepatocyte nucleus (HN) and hepatocyte cytoplasm (HC) diameters. Scale bar = 10 µm.

3.3. Digestive Enzymes

Acidic proteases showed no significant differences between treatments (Table 4; $p > 0.05$), while alkaline proteases and amylase showed significant increases in T18 treatment. The higher values of alkaline proteases for T18 are in consonance with the bigger digestibility of the protein demonstrated by this diet over H18 (Table 1). Because IM diets showed higher values of alkaline proteases than C, as well as lower acidic–alkaline ratios (non-statistically significant for H18), this supports the idea that insect-based diets suffered a more active digestion in the intestine. Contrarily, the work of Coutinho [23] showed a lower activity of total alkaline protease, trypsin, and lipase even on low inclusions of TM (defatted) for meagre (*Argyrosomus regius*). However, this work and the work of Guerreiro [22] showed that meagre might not be the best candidate to use IMs as a source of protein, which supports the point that differences between species must always be considered. In the present study, the increase in alkaline proteases could have been caused by the added effort of having to break the β(1–4) glycosidic bonds of chitin polymers, and especially its metabolites, because it was proven that chitinase activity is low in rainbow trout [90]. However, because chitinase (mostly located in the stomach) and chitobiase (mostly located in the intestine) activities were not measured, this would remain as a theory to encourage more future research.

Table 4. Digestive enzymes of rainbow trout fed experimental diets.

Digestive Enzymes (U/mg Protein)	C	H18	T18	SEM	*p*-Value
Acidic proteases	400.9	314.9	383.4	47.6	0.470
Alkaline proteases	82.2 ^{b}	117.0 ^{b}	263.5 ^{a}	23.4	0.001
Acidic–alkaline ratio	6.36 ^{a}	2.60 ^{a,b}	1.49 ^{b}	0.90	0.009
Amylase	81.5 ^{b}	85.1 ^{a,b}	139.5 ^{a}	15.2	0.043

C: control diet (no IM inclusion); H18: 18% HI inclusion; T18: 18% TM inclusion. $^{a, b}$ Show statistically significant differences among diets ($p < 0.05$); Values are expressed as mean ± standard error of the mean (SEM; n = 4 tanks per diet, 2 fish per tank).

The results for amylase activity follows closely the tendency of a previous study [32]. It has been described that amylase activity increases with the amount of carbohydrates in the diet [91]. Moreover, Rapatsa and Moyo already proved [92,93] that higher levels of *Imbrasia belina* meal increased the levels of amylase, which was conferred to the remaining vegetable contents of *Imbrasia belina*, more than to the intrinsic components of that insect. Vegetables are the most common substrates for the feeding of insects. As part of the private knowledge of the insect provider, these data are not available in the present study, but the results in the amylase activity could be due, on the one hand, to different feeding habits of the insects, and on the other hand, to the different levels of wheat meal between H18 and T18 diets.

3.4. Liver Intermediary Metabolism

No differences were found between treatments for any of the measured enzymes (FBPase, PK, GPT, GOT and GDH), which means that intermediary metabolism in the liver was mostly unaffected (Table 5). As stated previously, protein use (Table 3) was in general more efficient on T18 than on H18, which gives the idea that protein availability was better for fish fed with T18. The literature concerning the analysis of intermediary liver metabolism after a feeding trial with IMs is scarce, but supports the point that these ingredients do not disrupt the function of these enzymes [23,28,94,95]. In a previous experience [32] with similar diets and rearing conditions, but with 10% inclusion level of IMs, an increase in GOT activity was observed; in the present study, even though T18 showed a very similar trend (p-value = 0.059), the ANOVA did not reveal a significant difference. Considering that liver histology did not show differences either, this also supports the present data.

Table 5. Intermediary metabolism enzymes in liver of rainbow trout fed experimental diets.

Enzymes (mU/mg Protein)	C	H18	T18	SEM	p-Value
Fructose 1,6- biphosphatase (FBPase)	26.6	22.8	27.7	4.49	0.724
Pyruvate kinase (PK)	55.1	50.2	64.9	6.84	0.346
Glutamate pyruvate transaminase (GPT)	329.3	371.5	333.2	30.1	0.568
Glutamate oxaloacetate transaminase (GOT)	270.3	169.3	326.3	40.1	0.059
Glutamate dehydrogenase (GDH)	574.6	379.4	523.0	65.9	0.151

C: control diet (no IM inclusion); H18: 18% HI inclusion; T18: 18% TM inclusion. Values are expressed as mean ± standard error of the mean (SEM; n = 4 tanks per diet, 2 fish per tank).

3.5. Non-Specific Immune Status

The immune status was evaluated in different tissues. Tumour necrosis factor-alpha (TNF-α) was determined as a pro-inflammatory indicator [96] in distal intestine, as a first immune barrier from which an immune response can be initiated [97], and in skin mucus, for its role in innate immunity and fish health [98]. In addition, different parameters in plasma related to non-specific immune responses were determined.

No significant differences were found between treatments for TNF-α in distal intestine and skin mucus, or for lysozyme, esterase, anti-protease, alkaline phosphatase, peroxidase, or total immunoglobulins (IG) in plasma (Table 6).

Table 6. Non-specific immune parameters of rainbow trout fed experimental diets.

Immune Parameters	C	H18	T18	SEM	p-Value
TNF-α DI	0.43	0.36	0.59	0.08	0.189
TNF-α SM	0.75	0.37	0.52	0.11	0.105
Lysozyme	4.76	5.03	3.95	0.47	0.288
Esterase	1014.0	1114.9	1451.5	228.9	0.405
Acid phosphatase	882.1 [a]	791.2 [a,b]	581.9 [b]	53.9	0.009
Alkaline phosphatase	2350.4	2215.5	1780.8	291.6	0.392
Anti-protease activity	91.8	101.2	103.7	7.56	0.525
Peroxidase	0.58	0.69	0.47	0.08	0.223
Total Immunoglobulins	12.2	14.5	16.3	1.51	0.205

C: control diet (no IM inclusion); H18: 18% HI inclusion; T18: 18% TM inclusion. TNF-α D.I.: Concentration of Tumour Necrosis Factor alpha in distal intestine; TNF-α S.M.: Concentration of Tumour Necrosis Factor alpha in skin mucus expressed as µg/mL; Lysozyme activity expressed as µg/mL HEWL (Hen Egg White Lysozyme); Esterase, acid and alkaline phosphatases as mU/mg protein; anti-protease activity as U anti-protease/mg protein; peroxidase as U/mg protein; total IG as mg/mL. [a, b] Show statistically significant differences among diets (p < 0.05). Values are expressed as mean ± standard error of the mean (SEM; n = 4 tanks per diet, 2 fish per tank).

The only statistically significant difference was on acid phosphatase in plasma, where T18 showed the lowest level, being different to C ($p < 0.05$; Table 6). Acid phosphatase is related to tissue damage in other species [99,100]. IMs are known for causing varied effects on the immunological system of fish, while the precise mechanisms that produce them are still unknown [14,30,33,88]. In general, the immunostimulant effect of Ims is well accepted [30,101,102], and a frequent justification for this is the influence of chitin and its derivatives, together with the presence of antimicrobial peptides in insects [13,103]. However, a publication by Xu [104] described how the replacement of soybean oil with an ω-3 enriched insect oil modified the genetic expression of IL-1β, IL-10, and TNF-α on the liver and kidney of juvenile mirror carp, as well as the amount of serum lysozyme. Moreover, Kumar [88] carried out two parallel experiments, one based on the addition of HI meal, and one on the replacement of fish oil with insect oil, and described different results for the same immunological parameters and the same organs of rainbow trout. Because the immunological system is complex and multifactorial, the different components of IMs such as chitin, insect fat, or other that might not be considered, could lead to very different interactions with it. Even though these immunological benefits are usually attributed to IMs, its chitin, or both [30,101], concluding results will not be reached until the precise mechanisms involved are described.

3.6. Liver Antioxidant Status and Fish Welfare Indicators

With the exception of GPx activity, which was higher for C than for IM-based diets ($p < 0.05$), T18 showed an overall more active antioxidant status. There were no differences for G6PDH or GR. SOD activity was higher in T18 than in C and H18 ($p < 0.05$). Higher levels of CAT activity were highlighted in T18 than in H18, and lower levels of MDA are described in T18 than in C diet ($p < 0.05$). No differences were found between treatments for glucose or lactate plasmatic levels (Table 7).

Table 7. Liver antioxidant performance and fish welfare plasmatic parameters of rainbow trout fed experimental diets.

Antioxidant Enzymes and Lipid Peroxidation	C	H18	T18	SEM	*p*-Value
Superoxide Dismutase (SOD)	208.1 ^{b}	209.7 ^{b}	273.3 ^{a}	15.3	0.023
Catalase (CAT)	186.5 ^{a,b}	167.7 ^{b}	216.7 ^{a}	11.6	0.043
Glutathione Peroxidase (GPx)	12.2 ^{a}	10.1 ^{b}	9.85 ^{b}	0.32	0.001
Glutathione Reductase (GR)	7.02	7.82	6.36	0.46	0.136
Glucose-6-phosphate dehydrogenase (G6PDH)	43.2	41.5	41.6	2.71	0.887
Malondialdehyde (MDA)	21.3 ^{a}	15.7 ^{a,b}	11.1 ^{b}	2.40	0.043
Fish welfare indicators					
Glucose	3.33	3.69	3.23	0.19	0.319
Lactate	1.85	2.38	2.53	0.32	0.540

C: control diet (no IM inclusion); H18: 18% HI inclusion; T18: 18% TM inclusion. SOD and CAT expressed as U/mg protein; GPX, GR and G6PDH as mU/mg protein; MDA as nmol/g tissue; Glucose and lactate as mmol/L. $^{a, b}$ show statistically significant differences among diets ($p < 0.05$). Values are expressed as mean ± standard error of the mean (SEM; $n = 4$ tanks per diet, 2 fish per tank).

The antioxidant system is a complex biochemical structure composed of several molecules and enzymes that fight against the derived toxicity of reactive oxygen species (ROS) resulting from cellular metabolic processes. Roughly, SOD, CAT, and GPx are involved as direct defensive mechanisms against ROS, while GR regenerates the substrate of GPx (glutathione) and G6PDH works on the maintenance of this system by providing NADPH, which is used as a fuel to allow the activity of GR. An imbalance between ROS production and antioxidant mechanisms derivate in oxidative stress, resulting in cellular membrane lipids damage. MDA is a product formed from the breakdown of polyunsaturated fatty acids due to lipid peroxidation, and it may be used as a marker of

cellular damage [105]. Because all these molecules work together to prevent oxidative injury, it is not strange to find coincidences in their activities, such as the ones shown between SOD and CAT, higher on T18, as well as its consequential MDA decrease on T18. This would also be in consonance with the previously mentioned results of plasmatic acid phosphatase, because a more efficient antioxidant system should be reflected on a lesser amount of cellular damage. In general, the current bibliography strongly supports the idea that IMs help to prevent the derived toxicity of oxygen in two different ways: indirectly, by enhancing the antioxidant system, or directly, by preventing the oxidative damage itself. The first case can be easily recognized in those experiences where higher activities of antioxidant elements are highlighted, ideally, but not always, followed by a consequential decrease in the concentration of oxidative damage indicators (typically, MDA) when a significant amount of IM is added to the diet [21,30–32]. The second case can be more complicated, because the mechanisms that regulate the reduction of oxidative damage through the addition of IMs are not yet well known. As previously stated for the immunological system, a frequent hypothesis used to justify this involves the activity of chitin and its derivates in fish physiology, because it was described that this molecule could produce a direct scavenging effect on radical species, as well as an increase in intracellular glutathione [29]. The work of Moutinho [20] described a decrease in both SOD and CAT activities and a lower concentration of MDA on European seabass liver after an increasing amount of HI was added to the diet, suggesting this preventive effect. Moreover, the work of Sánchez-Muros on tilapia [106] described an increase in liver SOD activity, muscle ROS, and intestine ferric-reducing antioxidant power for a diet based on FM and soy meal, while two experimental diets with TM meals produced the opposite effects, even though one of them had the same amount of soy meal as the first, giving again the idea of a preventive effect. Interestingly, the work of Xu [104] highlighted an increase in SOD activity in the liver of juvenile mirror carp after the administration of HI oil, which suggests that chitin might not be the only element involved in the enhancement of the antioxidant system of fish after using whole IMs.

Glucose and lactate are known to be indicators of animal welfare, because their levels in plasma are increased after stressful situations [107–109]. Other trials based on the evaluation of IMs as ingredients for fish compared the levels of plasmatic glucose, but in general, not many changes have been described [110,111]. The present study matched this case; on the one hand, this means that the fish homeostasis was correct and compensated even in those cases where other indicators (acid phosphatase and MDA) suggested the presence of tissue damage; on the other hand, IMs did not affect either of these animal welfare indicators in a positive way.

3.7. Proximate Composition of the Fillet

The proximate composition of rainbow trout fillets was appointed in Table 8. Moisture levels were lower for T18 than for C. Protein and ash levels were higher for insect-based treatments than for C diet, while fat showed no significant differences. Despite the lower values of phosphorus in IMs, the phosphorus content in the fillets was similar among treatments, so IMs were able to satisfy the nutritional requirements.

In general, dry components were higher on fillets of fish fed with insect-based diets. Several studies have evaluated the composition of rainbow trout fillets after a growth trial with HI and TM IMs. Some of them described no differences in fish fillet compositions [62,67,112], while others described small changes in raw protein or lipids, the decrease on these parameters being more frequent than the opposite trend [61,65,113]. However, all the cited manuscripts describe experiences with bigger sizes of rainbow trout than those used for the present study. Our previous study [32] did not show any differences on fillet protein, fat, or ash, which means that the increased inclusion of HI and TM in the diets (18% vs. 5/10%) may have played an important role on these changes. Considering this, it is possible that smaller fish could have dealt differently with higher amounts of IMs, contrary to what was described for bigger fish.

Table 8. Proximate composition of fillets of rainbow trout fed experimental diets.

Proximate Composition of the Fillet (Wet Basis)	C	H18	T18	SEM	*p*-Value
Moisture (%)	77.6 [a]	77.2 [a,b]	76.4 [b]	0.24	0.017
Protein (%)	18.2 [b]	19.6 [a]	19.5 [a]	0.24	0.006
Fat (%)	1.09	1.87	1.52	0.21	0.070
Ash (%)	1.29 [b]	1.42 [a]	1.38 [a]	0.01	0.0001
Calcium (mg/Kg)	325.4	418.0	400.0	46.2	0.365
Phosphorus (g/Kg)	2.98	2.92	2.96	0.06	0.813

C: control diet (no IM inclusion); H18: 18% HI inclusion; T18: 18% TM inclusion. [a, b] Show statistically significant differences among diets ($p < 0.05$). Values are expressed as mean ± standard error of the mean (SEM; $n = 4$ tanks per diet, 2 fish per tank).

4. Conclusions

This study shows the importance of adequately selecting a type of IM before its inclusion as an ingredient in aquafeeds. Although in terms of absolute values for growth performance, the use of HI or TM in feeds for rainbow trout was efficient, fish fed with TM grew better than fish fed with HI. These differences have been marked by the higher use of the protein and more active digestive function, supported with intestinal histological changes observed, particularly the increase in villi height for T18. It is also remarkable that a small increase in enterocyte height was described for insect-based diets, which could be related to the different absorption of insect fat.

No changes were noticed for liver histology or intermediary metabolism. The antioxidant and immunological systems suffered a slight activity improvement for insect-based diets reflected on the decrease in tissue damage indicators (MDA and acid phosphatase), but this did not modify the overall health and welfare status of fish. Although more research is encouraged to isolate and identify the specific physiological mechanisms that make IMs improve the performance of both the antioxidant and the immunological systems of fish, this study supports the idea that IMs act as potential functional ingredients.

Minor changes in the composition of the fillets were observed, with a higher amount of protein in fish fed with insects. More research is encouraged to elucidate the long-term feeding effects.

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Data Availability Statement: The data presented in this study are available in the article.

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CHAPTER 5 – Study 3

DIFFERENT DIETS BASED ON YELLOW MEALWORM (*Tenebrio molitor*): FACING THE DECREASE OF OMEGA-3 FATTY ACIDS IN FILLETS OF RAINBOW TROUT (*Oncorhynchus mykiss*)

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Abstract

Aquaculture is still struggling with sustainability due to the use of fishmeal, and insects are one solid alternative. However, insects have very low content in long chain omega-3 polyunsaturated fatty acids, which is why insect-fed fish tend to reflect this in the composition of their fillets. In this study, 500 rainbow trout (*Oncorhynchus mykiss*) were fed until fish reached a commercial size, evaluating two strategies that aimed to solve the omega-3 problem previously mentioned: I) by using a diet based on partially defatted insect meal from yellow mealworm (*Tenebrio molitor*), and II) other two diets with the addition of an experimental algal oil rich in omega-3. Growth was unaffected, as well as texture and organoleptic profile of the fillets. Lightness, brightness and colour of the fillets were slightly modified by the experimental diets. Digestibility of the protein, body indices and butchering yield showed a minor but consistent trend. The omega-3 problem was solved, but the omega-3 sparing effect of fish caused lipid accumulation in fillets and liver, followed by a mild increase in oxidative damage, which was followed by the level of phosphatases in skin mucus. The histology of the intestine showed signs that insect meals could be softening a mild inflammatory response caused by control diet. The microbiota suffered several changes which could be associated with the different amino acid and fatty acid composition of the diets. According to the results, the strategy of using a diet based on

defatted insect meal was the most promising alternative to overcome the problem. More research is encouraged to evaluate the fatty acid profile of the liver in similar feeding trials.

1. Introduction

Aquaculture is one of the fastest developing industrial sectors related to food production [1], however, it is due to this same growth that some fundaments of this industry become a big liability. Even though their growth ratios make fish farming more efficient than conventional land-based animal species, they need higher amounts of protein to satisfy their nutritional needs in the short term [2]. The biggest part of these protein requirements have been satisfied traditionally through the use of fishmeal (FM), mostly from extractive fishing practices, so the use of this ingredient to satisfy the protein demands of this sector is not sustainable.

A good counterpart is that this problem has been known and addressed for several years now, and the usage of protein alternatives to FM in fish feedings is a reality nowadays. However, this has led to other secondary problems directly related to these alternatives. The most typical case is that of vegetable-sourced ingredients like soybean meal, which are known for having antinutrients in their composition [3] and for disrupting a correct and healthy status of the fish gastrointestinal tract (GIT) [4-6] among other effects. Even though these consequences are now very well known, which allows the use of these ingredients working around their disadvantages [7,8], the search and study of other sources of protein is still a good way to go for aquaculture research in order to offer a higher diversity of ingredients.

Insect meals (IMs) appear as one of the most promising among these alternatives. The production of insects on an industrial scale can be environmentally friendly due to their feed and growth ratios, the high speed of their reproduction cycles, and the high adaptability of many of these animals to different feeding substrates [9,10]. Also, once they are processed and turned into ingredients they count with other advantages, such as the good quality of their protein [11,12] and the possibility of using them as functional ingredients [13-15]. However, and as for many other ingredients, insects also present some disadvantages. Most terrestrial insects have an extremely low proportion of long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA) within their composition [11], an important detail that is reflected in the fatty acid (FA) profile in fillets of insect-fed fish [16-20]. Depending on the situation, this might not be a big concern because it is known that this lower level of n-3 in the fish fillet should still make their fish lipid quality indices stay between reasonable levels to be considered as healthy products [21,22]. Nevertheless, one of the strongest points of fish as a component of a healthy diet is its FA profile, so the possibility of worsening it through the use of novel ingredients should not be left unchecked.

This problem concerning insects as a key ingredient for fish feeding has already been addressed through different strategies. The most typical among them is to perform a defatting process on the IM to reduce the amount of insect fat in the feed, which is a widespread practice in current studies [23-25]. Another possibility is to make use of the already mentioned ability of insects to use different feeding substrates, as well as the capacity to modify their internal composition according to these substrates. Indeed, there are experiments which describe “enriched” IMs that came from insects fed on substrates rich in n-3 such as fish by-products, with

promising results [26-28]. Last but not least, there is also the possibility of simply readjusting the feed formula to modify the lipid sources, using ingredients with a higher concentration of essential LC n-3 PUFA like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [29].

As for many other changes in the diet, and as for any other novel ingredient, different interactions and changes in physiology should be expected and not underestimated. As previously stated, IMs may act like functional ingredients due to their proven capacity to boost the antioxidant and the immunological systems, and also improving the general state of the intestinal microbiome [30-32]. On the other hand, changes in the balance of different FAs, and especially those with high peroxidability indices such as LC n-3 PUFA could also alter fish physiology [33]. Following the line of previous research [18,34], the present study tried to unify as many of the mentioned ideas as possible using two different strategies to solve the n-3 problem of insect-fed fish growing up to commercial size by: I) reducing the insect fat proportion; II) adding an ingredient rich in LC n-3 PUFA. Several variables were measured to follow the repercussions of these diets, from pure physiological parameters such as growth, protein digestibility, histomorphology, antioxidant, immunological or microbiota analyses, to indirect effects such as the filleting yield, the composition of the fillet and the perception of the final product.

2. Materials and methods

2.1. Experimental diets

The dried larval stage of yellow mealworm (*Tenebrio molitor*; TM) was used for the diets of this study in the form of processed insect meals (IMs) [**Table 1**]. Five isoproteic (≈48.9 %) and isolipidic (≈18.5 %) diets were formulated with the following principles: a control diet (C) with no IM based on fishmeal (FM) as the main source of protein; one diet with a 50 % FM replacement using whole TM meal (T; 18 % diet inclusion; Tebrio, Spain); one diet similar to the previous one, but using defatted TM meal (dT; 18 % diet inclusion; provided by Ÿnsect, France); two diets similar to T, but with an increasing level of fish oil replacement with an experimental algal oil (EO) containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (TO1 and TO2; 3.09 and 7.24 % inclusion, respectively; supplier decided to stay anonymous). The concentration of the EO was adjusted to match the theoretical concentration of EPA and DHA, being TO1 equivalent to dT, and TO2 equivalent to C [**Table 2a**]. Diets were enriched with methionine and lysine to satisfy the requirements of the fish [35,36], were manufactured by the Experimental Diets Service of the University of Almería (Almería, Spain), and extruded as pellets of 3 and 4 mm, for the different stages of fish. The dough passed through a two screw laboratory extruder (Evolum 25, Clextral, France). The extruder barrel had four sections, with a temperature per section of (inlet to outlet) 100 ºC, 95 ºC, 90 ºC and 85 ºC. Pellets stayed for 24 h in a drying chamber, at 30 ºC (Airfrio, Spain) and were stored in sealed plastic bags at -20 °C until use.

2.2. Experimental animals and rearing conditions

The private company Mundova (Albacete, Spain) provided rainbow trout (*Oncorhynchus mykiss*) eggs to the experimental facilities of the Aquaculture Research Centre of "Instituto Tecnológico Agrario de Castilla y León" (ITACyL), where they were hatched and reared until

they were ready for the experiment. Afterwards, a total of 500 female fish were allocated into 20 cylindrical fiber-glass tanks (500 L; four replicates per treatment; 25 fish per tank) in a recirculating system, and stayed in acclimation during three weeks before the beginning of the growth trial (initial body weight of 46.1 ± 0.1 g). The recirculating system was maintained under controlled conditions as follows: water temperature of 14.8 ± 0.7 °C, water dissolved oxygen of 7.8 ± 0.7 mg/L, room photoperiod of 12 h light: 12 h dark, ammonia < 0.1 mg/L and nitrite < 0.1 mg/L (daily analyses).

Table 1. Proximate and amino acids compositions of fishmeal and insect meals.

Proximate Composition	***Fishmeal LT94***	***T. molitor***	***Defatted T. molitor***
Moisture (%)	5.0	6.5	2.6
Crude protein (%)	69.4	52.8	70.7
Crude lipid (%)	12.3	28.5	8.9
Chitin (%)	-	3.2	5.5
Ash (%)	16.0	2.91	3.96
Calcium (Ca, g/kg)	38.0	0.51	1.10
Phosphorus (P, g/kg)	26.0	6.5	8.2
Ca:P ratio	1.46	0.08	0.13
Amino acid composition (g/100 g insect meal)			
Asp (aspartate)	7.86	4.03	5.53
Thr (threonine)	3.21	1.85	2.54
Ser (serine)	2.78	2.09	2.84
Glu (glutamate)	9.09	5.52	7.48
Pro (proline)	3.61	3.22	4.05
Gly (glycine)	3.60	2.69	3.46
Ala (alanine)	5.2	3.62	4.70
Cys (cysteine)	0.50	0.40	0.43
Val (valine)	5.24	3.40	4.32
Met (methionine)	1.68	0.68	0.93
Ile (isoleucine)	2.90	2.18	2.92
Leu (leucine)	4.5	3.55	4.82
Tyr (tyrosine)	2.35	5.89	8.06
Phe (phenylalanine)	2.93	2.10	3.18
His (histidine)	1.23	1.60	2.22
Lys (lysine)	5.95	3.44	4.68
Arg (arginine)	3.48	2.59	3.39

2.3. Growth trial, sample collection and butchering yield

The growth trial lasted 89 days. Fish were fed daily by hand (9 a.m.) until apparent satiation was reached (3 % tank biomass as maximum feed intake). Feed intake and mortality were monitored daily. A simple biometry procedure was carried out every 21 days to measure and weigh the fish after being fasted for one day, using a scale (± 0.1 g) and a graduated

icthyometer (± 0.1 mm), and being previously anesthetized with tricaine methanesulfonate (MS-222; 80 mg/L).

Table 2a. Formulation and proximate composition of experimental diets.

Ingredients (%; on dry basis)	C	T	dT	TO1	TO2
Fishmeal LT94 [1]	36.78	18.28	18.48	18.28	18.28
TM meal [2]	-	19.05	-	19.05	19.05
Defatted TM meal [3]	-	-	18.29	-	-
Enriched omega-3 oil	-	-	-	3.09	7.24
Wheat gluten [4]	11.05	12.41	10.65	12.41	12.41
Soybean protein concentrate [5]	15.09	16.4	15.41	16.4	16.4
Wheat meal [6]	16.16	16.98	16.19	16.98	16.98
Soybean lecithin [7]	1.27	0.48	1.27	0.48	0.48
Fish oil [8]	11.87	8.68	11.9	5.6	1.45
Vitamin and mineral premix [9]	1.95	1.93	1.95	1.93	1.93
Goma guar [10]	1.95	1.93	1.95	1.93	1.93
Blood meal [10]	3.89	3.86	3.90	3.86	3.86
Methionine [10]	0.2	0.5	0.5	0.5	0.5
Lysine [10]	-	0.1	0.1	0.1	0.1
Proximate composition (%; on dry basis)	**C**	**T**	**dT**	**TO1**	**TO2**
Crude protein	49.19	48.76	48.98	49.25	48.23
Crude lipid	17.80	18.58	17.86	18.99	19.12
Crude fibre	0.97	2.15	2.35	2.25	2.47
Ash	8.63	8.49	6.31	6.22	6.12
Calcium (Ca)	0.43	0.24	0.17	0.18	0.20
Phosphorus (P)	0.31	0.24	0.24	0.24	0.24
Ca:P ratio	1.38	1.0	0.73	0.77	0.86

[1] Norsildemel, Norway.
[2] Tebrio, Spain.
[3] Ÿnsect, France.
[4] 78 % crude protein (Lorca Nutrición Animal SA, Spain).
[5] Soycomil, 60 % crude protein, 1.5 % crude lipid (ADM, Poland).
[6] Local provider (Spain).
[7] P700IP (Lecico, DE).
[8] AF117DHA (Afamsa, Spain).
[9] Lifebioencapsulation SL: Vitamin and mineral premix (g/100g feed unless otherwise specified): vitamin A 2,000,000 UI; vitamin D3: 200,000 UI; vitamin E: 1.2; vitamin K3: 0.26; vitamin B1: 0.3; vitamin B2: 0.3; vitamin B6: 0.2; vitamin B9: 0.15; vitamin B12: 0.001; vitamin H: 0.03; inositol: 5; betaine: 5; calcium pantothenate: 1; nicotic acid: 2; Co: 0.006; Cu: 0.09; Fe: 0.06; I: 0.005; Mn: 0.095; Se: 0.0001; Zn: 0.075; Ca: 19; K: 2.4; Na: 4.1.
[10] Lorca Nutrición Animal SA, Spain.
C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched oil (EO); TO2: T diet supplemented with 7.24 % of EO.

A modified Guelph method [37] was followed during the last days of the experiment (daily, approximately two weeks) to collect faeces from settling columns, one per tank; the faeces were frozen and kept at -80°C until they were analysed. After the end of the feeding trial, a general biometry of all fish was carried out to measure growth. The rest of the sampling procedure was

scheduled to take place during the course of a week. Two fish per tank were taken in order to get blood, liver, distal intestine, pyloric caeca, skin mucus and dorsal fillet samples for different analyses. Other measures were taken during the process to analyse filleting yield and somatic indices. All fish were chosen at random from the tanks and sacrificed by an overdose of MS-222 (300mg/L). Two exceptions were made for the previously mentioned: first, three fish per tank were taken for microbiota analyses (distal intestine digesta samples); second, due to logistic reasons, four fish per treatment were taken for sensorial analyses of whole raw fish and fillets, which was performed in the following two hours, and other four fish for cooked fillets. In this last case, the fish were sacrificed using ice, and their fillets were kept at -20 ºC until sensorial analyses.

Table 2b. Amino acid and mineral composition of experimental diets.

Amino acid composition (g/100 g feed)	**C**	**T**	**dT**	**TO1**	**TO2**
Asp (aspartate)	3.32	3.56	3.35	3.1	3.12
Thr (threonine)	1.48	1.56	1.48	1.31	1.36
Ser (serine)	1.76	1.87	1.91	1.65	1.79
Glu (glutamate)	7.15	7.64	7.41	7.44	7.44
Pro (proline)	2.46	2.51	2.67	2.74	2.61
Gly (glycine)	1.92	2.2	1.91	1.81	1.8
Ala (alanine)	2.01	2.06	2.18	1.95	1.94
Cys (cysteine)	0.46	0.66	0.46	0.43	0.44
Val (valine)	2.1	2.07	2.16	2.25	2.1
Met (methionine)	1.01	1.15	1.23	1.18	1.13
Ile (isoleucine)	1.5	1.69	1.45	1.56	1.44
Leu (leucine)	3.0	2.99	3.02	2.9	2.88
Tyr (tyrosine)	1.63	1.78	2.94	2.12	2.42
Phe (phenylalanine)	2.07	2.19	2.01	1.96	1.95
His (histidine)	1.09	1.02	1.05	1.05	1.04
Lys (lysine)	2.94	3.66	2.77	2.7	2.66
Arg (arginine)	2.1	2.35	2.08	2.01	2.0
Mineral composition (g/100 g feed)	**C**	**T**	**dT**	**TO1**	**TO2**
Sodium (Na)	0.25	0.14	0.14	0.13	0.14
Magnesium (Mg)	0.07	0.08	0.08	0.08	0.07
Silicon (Si)	0.11	0.11	0.09	0.10	0.13
Phosphorus (P)	0.29	0.22	0.22	0.22	0.22
Sulfur (S)	0.20	0.21	0.20	0.22	0.21
Chlorine (Cl)	0.34	0.26	0.23	0.25	0.25
Potassium (K)	0.34	0.38	0.44	0.46	0.45
Calcium (Ca)	0.40	0.22	0.16	0.17	0.19

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO.

Samples for enzyme determinations were kept in liquid nitrogen during the sampling procedure and frozen at -80 ºC until their individual analyses. Samples for histomorphology analyses were

fixed in 4 % buffered formalin for 48 h before dehydration and processing. Digesta samples for microbiota analyses were frozen at -80 ºC until their individual analyses.

The Directive of the European Union Council and the Spanish Government [38,39] was followed for the care and handling of the fish. The Bioethical Committee of "ITACyL" approved this experiment (Authorization number: 2017/19/CEEA).

Table 2c. Fatty acid composition of experimental diets.

Fatty acid composition (g/100 total FA)	C	T	dT	TO1	TO2
Myristic (MYR; C14:0)	3.68	3.26	2.55	3.16	3.2
Palmitic (PA; C16:0)	21.13	19.37	17.35	20.75	22.74
Palmitoleic (POA; C16:1n-7)	4.68	2.97	3.56	3.02	2
Stearic (STE; C18:0)	5.67	4.78	4.96	3.95	2.87
Oleic (OA; C18:1n-9)	13.67	23.05	16.17	20.47	17.14
Vaccenic (VA; C18:1n-7)	2.6	1.54	1.42	0	0
Linoleic (LA; C18:2n-6)	8.77	18.69	13.75	15.31	15.21
Linolenic (ALA; C18:3n-3)	1.12	1.33	1.56	0.95	0.87
Stearidonic (SDA; C18:4n-3)	0.82	0.44	0.89	0.61	0.51
Eicosenoic (GOA; C20:1n-9)	0.98	0.76	1.75	1.14	0.56
Arachidonic (ARA; C20:4n-6)	1.66	1.22	1.56	1.17	1.26
Eicosapentaenoic (EPA; C20:5n-3)	8.31	3.92	6.42	6.33	8.51
Docosapentaenoic (DPA; C22:5n-3)	1.33	0.89	1.49	1.08	1.12
Docosahexaenoic (DHA; C22:6n-3)	18.79	14.05	19.07	16.1	19.2
Other (up to 100 %)	6.79	3.73	7.5	5.96	4.81
∑Saturated fatty acids (SFA)	30.48	27.41	24.87	27.86	28.81
∑Monounsaturated fatty acids (MUFA)	21.93	28.32	22.91	24.63	19.7
∑Polyunsaturated fatty acids (PUFA)	40.8	40.54	44.73	41.56	46.67
∑Omega-3 (n-3)	30.37	20.63	29.42	25.07	30.2
∑Omega-6 (n-6)	10.43	19.91	15.31	16.49	16.47
∑Omega-6/∑Omega-3 (n-6/n-3)	0.342	0.97	0.52	0.66	0.55
∑Total fatty acids	100	100	100	100	100

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO.

2.4. Final product quality

2.4.1. Instrumental texture and colour

Samples were thawed overnight at 5 ºC. Texture was analysed using a TA-XT2i Texture Analyzer (Stable Micro Systems, Spain) equipped with a 5 kg load cell and two different probes, one cylindrical (2 mm diameter) for a brittleness test, and one spherical (2.5 cm diameter) for a complete texture profile analysis (TPA). Texture parameters were determined as described by Rosenthal [40] in such conditions as at pre-test speed 8 mm/s, test speed 1 mm/s, post-test speed 10 mm/s, distance of 5 mm, trigger type 1 g, time 5 seconds. A Chroma Meter CR 400 (Minolta, Spain) was used for colour determination, measuring 10 random points per fillet that were

expressed as CIELab coordinates: lightness (L*) is expressed on a 0-100 scale of black to white; scale of red (+) or green (-) colour, expressed as redness (a*); scale of yellow (+) or blue (-) colour, expressed as yellowness (b*); chroma value (C*ab = (a*2+b*2)1/2) as an expression of the intensity and clarity of the colour; hue (H°ab= arctan (b*/a*)) is the name of a colour as it is found in its pure state on the spectrum, and is expressed in degrees°, ranging from 0° (red) through 90° (yellow), 180° (green), 270° (blue) and back to 0° [41].

2.4.2. Direct sensorial analyses

The sensorial evaluation of raw rainbow trout was interpreted in two stages. The first one, for raw fish and fillets, was analysed by thirteen panellists as was previously described by Tomás-Almenar *et al*. [42], on whole fish and on fish fillets in four fish per diet (two for whole fish, and two for fish fillets). Briefly, for the sensorial assessment of fish fillets, a quantitative descriptive analysis of four attributes (acceptability, colour, texture and odour) was performed using an equal-interval scale, where the panellists had to place a vertical mark across a 10 cm horizontal line. Whole fish were evaluated for external characteristics using a scoring system similar to the QI method, including 9 parameters related to 3 main attributes (general appearance, eyes and gills), and the total score for all parameters (QI from 0 to 20) was used as another attribute to evaluate the perception of the panellists. Fillets of four fish per diet were used for cooked fillet analyses. The fillets were cooked in an oven at 130 ºC during 20 min. The most cranial and caudal sections of the fillets were discarded before cutting the fillets in a total of three individual portions. After proper labelling, the different portions were given to eight trained panellists, making sure that all of them could analyse a minimum of two different samples from each treatment. The variables analysed are described in **Table 10**. Informed consent was obtained from all panellists involved in the study.

2.5. Analytical determinations

2.5.1. Chemical analyses

The moisture and fat content of both IMs, the five diets and fish fillets were analysed following AOAC methods [43], while a modified Dumas method [44] was used to determine their protein content, using a nitrogen analyser (FP 528, LECO, St. Joseph, USA). The conversion factors for protein analyses were 6.25 for feeds and faeces, and 4.75 for TM [45]. Chitin was isolated from IMs, washed with acetone, dried and weighed as described by the method of Gamage and Shahidi [46]. Samples of TM meal and defatted TM meal were hydrolysed at 110 °C, for 22 h, with 6 N HCl [11]. Ion-exchange liquid chromatography and postcolumn continuous reaction with ninhydrin (Biochrom 30; United Kingdom) was used for the amino acid (AA) determination. Tryptophan was not determined. For the mineral composition of feeds, the pellets were prepare using a Mignon SS hydraulic press (Nannetti, Italy), and the quantification of elements was carried out with an X-ray fluorescence equipment (Bruker S4 Pioneer, Bruker, Spain), through wavelength dispersion; results were analysed with the Plus EVALUATION program of the Spectra plus package (Bruker, Spain). For the case of fillets, calcium (Ca) was determined with X-ray fluorescence method of Dispersive Energy, as described by Pessoa *et al*. [47], and phosphorus determination was carried out by molecular absorption spectrophotometry

(UV/Vis UV2, UNICAM, Cambridge, UK) according to ISO standards [48]. Apparent digestibility coefficient of the protein (ADC_{prot}) was determined using acid-insoluble ash as a marker in feeds and faeces [49].

2.5.2. Fatty acid determination

The fatty acid (FA) profile of diets and fish fillets was analysed following the method described in previous studies [50,51]. After a direct derivatization to FA methyl esters (FAMEs), and as described by [52] these FAMEs were analysed in a Focus GC (Thermo Electron. Cambridge. UK) equipped with a flame ionisation detector and an Omegawax capillary column (30 m x 0.25 mm i.d. x 0.25 μm film thickness; Supelco. Bellefonte. USA). The retention times of known FAME standards (PUFAs No. 1, 47,033 from Sigma, St. Louis, MO, USA) were used to identify the peaks of the analysed FAMEs, and methyl pentadecanoate (15:0; 99,5 % purity; 76,560 Fluka; Sigma, St. Louis, MO, USA) was used as internal standard to estimate the FA contents. The method described by Cladis [53] was followed to estimate the relative retention factors of each FA. Peroxidability index was calculated as described by García-Márquez *et al*. [54].

2.5.3. Liver intermediary metabolism

Nine volumes of icecold 100 mM Tris-HCl buffer pH 7.8 containing 0.1 mM EDTA and 1 g/kg (v/v) Triton X-100 were used to individually homogenize liver samples, on ice. Samples were then centrifuged for 30 min at 30,000 g (4°C). Aliquots were separated from the supernatants and kept at -80°C for the following enzyme assays. The enzymatic activity of fructose 1,6-bisphosphatase (FBP_{ase}; EC 3.1.3.11), pyruvate kinase (PK, EC 2.7.1.40), glutamate pyruvate transaminase (GPT; EC 2.6.1.2), glutamate oxaloacetate transaminase (GOT; EC 2.6.1.1), and glutamate dehydrogenase (GDH; EC 1.4.1.2) was measured following the method of Furné *et al*. [55], at 25 °C, monitoring the changes in absorbance with a PowerWaveX micro-plate scanning spectrophotometer (Bio-Tek Instruments, USA). The method described by Bradford [56] was followed to quantify the concentration of soluble protein in tissue homogenates, using as standard bovine serum albumin.

2.5.4. Liver antioxidant status and fish welfare indicators

In order to evaluate the antioxidant status of the fish, and following the method of Pérez-Jiménez *et al*. [57], the activity of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPx, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), and glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) were analysed monitoring the absorbance changes with a PowerWaveX microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). The optimal substrate and protein concentrations for the measurement of maximal activity for each enzyme were established by preliminary assays. For NADH/NADPH, DTNB, and H_2O_2, the millimolar extinction coefficients were, respectively, 6.22, 13.6 and 0.039/mM·cm. For SOD activity, one unit was defined as the amount of enzyme necessary to inhibit half of the ferricytochrome C reduction rate. For the rest of the enzymes, one unit of activity was defined as the amount of enzyme needed to transform 1 mol of substrate in one minute. All analyses were carried out at 25 °C. To complete the redox status analysis, the

level of lipid peroxidation was also measured through the quantification of malondialdehyde (MDA). Thiobarbituric acid reacts in the presence of MDA, producing coloured thiobarbituric acid reacting substances.

Commercial kits were used to analyse glucose and triglyceride levels in plasma, following the manufacturer instructions (Glucose-TR, ref. 41011; Triglycerides-LQ, ref. 41030, Spinreact, Spain), and the absorbance was measured in 96-well microplates with a microplate reader (ELx800TM; Bio-Tek Instruments, Winooski, VT, USA).

2.5.5. Non-specific immune status

The non-specific immune status of the fish was assessed as follows: lysozyme, antiprotease, acid and alkaline phosphatases, and peroxidase activities, together with immunoglobulins concentration, were measured in plasma; acid and alkaline phosphatases, peroxidase, esterase and carbonic anhydrase activities were measured in skin mucus.

A turbidometric method [58] with *Micrococcus lysodeikticus* (Sigma, St. Louis, MO, USA) was used to measure lysozyme activity in plasma. The reaction was carried out for 20 min at 35 °C. Activity was expressed as U/mL, and one unit of activity was defined as the amount of enzyme that catalysed a decrease in absorbance of 0.001 per minute at 450 nm.

The method described by Mashiter and Morgan [59] was followed to measure total esterase activity in skin mucus, at 25 °C. The chosen substrate was P-nitrophenyl acetate (0.8 mM), and acetazolamide (1.6 mM) was used as the inhibitor of carbonic anhydrase activity. Samples were then incubated for 10 min, and the increase of absorbance was measured for 5 min at 405 nm. The activity was expressed in U/mg protein (1 unit was defined as 1 μmol of substrate transformed per minute).

Antiprotease activity was measured in plasma following the method of Thompson *et al*. [60]. The variation of optical density (410 nm, for 30 min) was used to quantify the production of 4-nitroaniline, using the activity of trypsin in absence of plasma as control (CAS 90002-07-7, Acofarma, Spain). The activity was expressed in U/mg protein (1 unit was defined as the amount of enzyme that inhibits by 50 % the control reaction).

The activity of acid and alkaline phosphatases in both plasma and skin mucus was determined following the method of Huang *et al*. [61]. To measure acid phosphatase, a buffer at pH 5 ($CH_3COOH/CH_3COOHNa$ 0.1 M, $MgCl_2$ 1 mM) was used, and a buffer at pH 10 ($NaHCO_3/NaOH$ 0.05 M, $MgCl_2$ 1mM) was used to measure alkaline phosphatase, while the chosen substrate for both reactions was P-nitrophenyl phosphate (Sigma, St. Louis, MO, USA). The measurements were performed at 405 nm, 37 °C and 30 min. The activity was expressed in mU/mg protein (1 unit was defined as the amount of enzyme required to transform 1 μmol of substrate per minute).

The method of Mohanty and Sahoo [62] was followed to determine the activity of peroxidase in both plasma and skin mucus. TMB (3, 30, 5, 50-Tetramethylbenzidine) as a 20 mM solution was used as the substrate, while standard samples without plasma/skin mucus were used as controls. After blocking the reaction for 2 min, samples were read at 450 nm. The activity was expressed in U/mg protein (1 unit was defined as the amount of enzyme required to transform1 μmol of substrate per minute).

The method described by Panigrahi *et al*. [63] was followed to determine total immunoglobulins in plasma. Immunoglobulins were precipitated by adding 12 % polyethylene glycol (PEG) to plasma samples (10 µL plasma, 40 µL of saline solution, and 50 µL of PEG), and separated from the total proteins to calculate the difference in untreated plasma. Protein content in untreated and PEG-treated plasma samples was determined and immunoglobulin content was calculated by difference. Protein content of samples was analyzed using the method of Bradford [56], with bovine serum albumin used as a standard.

2.6. Histomorphology

2.6.1. Samples processing

Increasing ethanol solutions (25, 50, 75 and 100 %) were used to dehydrate the fixed samples, which were then embedded in synthetic paraffin. A rotary microtome (FINESSE ME+ Thermo Scientific, Waltham, MA, USA) was used to obtain histological sections (3-4 µm). Samples were processed with hematoxylin and eosin technique. A light microscopy with graded objective lenses was used to evaluate five random regions per tissue sample, with an Olympus CX31 microscope and an Olympus EP50 microscope camera (Olympus, Barcelona, Spain).

2.6.2. Distal intestine and pyloric caeca histomorphology analyses

The followed protocol was very similar to the one described in Melenchón *et al*. [34]. Briefly, the chosen measurements for the quantitative analyses of distal intestine and pyloric caeca were: villi height and width, enterocyte height, widths of *stratum compactum*, muscular layer and lamina propria, this last one being measured at three different heights (apical, intermediate and basal lamina propria) to calculate a mean. Also, a subjective, qualitative analysis was carried out to evaluate the levels of lamina propria inflammatory infiltration and, loss of supranuclear vacuolization of enterocytes. These subjective parameters were evaluated as absent (-) mild (+), medium (++) or severe (+++) level.

2.6.3. Liver histomorphology analysis

The followed protocol was the same described in Melenchón *et al*. [34]. Briefly, hepatocyte nucleus and cytoplasm widths were measured as quantitative variables, while the levels of hepatocyte intranuclear vacuolization and liver inflammation (inflammation and necrosis focuses) were evaluated as qualitative variables.

2.7. Distal intestine digesta microbiota analysis

Frozen digesta samples were thawed on ice. DNA extraction was carried out following the instructions of the commercial kits QIAamp Fast DNA Stool Mini Kit and QIAamp PowerFecal DNA Kit (QIAGEN Iberia, Barcelona, Spain). A DNA purification was carried out after the extraction, using the QIAGEN DNA blood&tissue kit (QIAGEN Iberia, Barcelona, Spain), followed by a quantification with a Qubit fluorometer 4 (Fisher Scientific, Madrid, Spain). DNA samples were kept at -20 ºC until library preparation.

Microbiome diversity was studied following the methodology of Hernández *et al*. [64]. Primers described by Klindworth *et al*. [65] were used to amplify the variable region V3-V4 of 16S rRNA from the DNA samples, using the 16S metagenomic sequencing library protocol

(Illumina, San Diego, MA, USA). Libraries normalised and pooled at 4 nM were denatured with NaOH 0.2 N, and combined with PhiX (Illumina, San Diego, MA, USA) as control. Samples were sequenced with parallel synthesis technology in a MiSeq platform (Illumina, San Diego, MA, USA), using a 2x300 cycle V3 Kit, following the Illumina sequencing protocols. After 72 h, a volume of data of 10-20 Gb was obtained and analysed through bioinformatics. The data was filtered to obtain a phred score ≥ 30 (99.9 % sequence accuracy) with the program FASTQC [66].

QiimeReporter [67] was used to curate the data and obtain Amplicon Sequence Variants (ASVs). Afterwards, a naive Bayesian classifier [68] was used for the taxonomic assignation of ASVs, having SILVA database 138 as reference [69]. The open-source programming tool R [70] and RStudio interface [71] were used to obtain richness (Chao1) and diversity (Shannon and Simpson) indices.

2.8. Statistical analyses

Statistical analyses were carried out with the open-source programming tool R [70] making use of RStudio interface [71]. The analysis of variance (ANOVA) was performed to determine the effect of the diet, and the multiple comparison of the means was performed by Tukey test being statically different at p-*Value* < 0.05. Values are shown as mean ± standard error of the mean. A Principal Component Analysis (PCA) was used to represent the relationship between diet composition (in relation to FAs and AAs) and microbiota digesta composition at genus level. Previous to PCA, the variables were transformed to the same scale. Figures were created with Microsoft Excel 2019 and RStudio Build 382.

3. Results and discussion

3.1. Growth performance, protein use and biometric indices

There were no differences in growth performance among the assayed dietary treatments [**Table 3**]. T and TO2 showed a higher protein efficiency ratio than dT, while TO1 and TO2 showed a higher level of apparent digestibility coefficient of the protein (ADC_{prot}) than C, T and dT. Also, TO1 and TO2 showed a general tendency of having a lower intestinal somatic index (ISI) than the rest of the diets.

In a majority of cases, it is well accepted that insect meals (IMs) do not interfere with fish growth when their inclusion in the diet is not too high [19,72], especially for the case of salmonids, which seem to have good performances with these kind of diets [73]. There are, however, some minor discrepancies to this, because there are feeding trials that described disruptions in growth, especially for species like meagre (*Argyrosomus regius*) [74-76], or the opposite, with studies that described better growth performances with the inclusion of IMs [77-79]. Due to the high amount of variables involved, such as the fish and insect species, the feeding strategies of the insects, or even the different strains of the experimental animals, it is uneasy to give a definite conclusion of why this happens. However, and taking a broader scope on the topic, the idea that low to mid-levels of fishmeal (FM) replacement with IMs do not impair fish growth is quite solid for most cases nowadays.

It has been described that chitin might interfere with the digestibility of protein [80,81]. However, as stated above when talking about growth, there are also many cases like the one of this study where this did not happen, which aims to the idea that this phenomenon could be attenuated when the levels of chitin are low. The chitin of the experimental diets was not measured during this study, but the levels found in the different IMs, 3.2 % in full fat yellow mealworm (TM) and 5.5 % in defatted TM, suggest that even the highest value (dT diet) should be around 1 %. In our case, TO1 and TO2 showed higher ADC_{prot} than the rest of the diets. The results of ISI, lower in TO1 and TO2, were inversely correlated to those of ADC_{prot}. German and Horn [82] described that, from the point of view of evolution, a longer intestine is usually related to a lower digestibility of the diet; even though little is known about this fact when talking within the same species, it is possible that less digestible diets could lead to the development of a more active and bigger/longer intestine [83].

3.2. Final product quality

Butchering yield of the fish was not statistically affected by the experimental treatments [**Table 4**]. As an interesting detail, the results on ISI and ADC_{prot} followed a logical trend with those of dressing and filleting yield, even though there was not a statistical difference for these two results.

The proximate composition of the fillets suffered a significant change in the levels of fat, and a compensatory change in moisture [**Table 5**], the levels of fat being higher for T and TO1 than for C, while dT and TO2 stayed within intermediate levels. The most probable cause for this is that the presence of insect fat interfered with the lipid deposition in muscle, as well as in liver (discussed further at a later point of this manuscript). The deposition of lipids in the muscle of insect-fed fish generates an intense controversy within the scientific literature, as there have been cases of increases [84-86], decreases [26,87,88], and no changes [89-91] for this variable. It is known that fish physiology counts with different metabolic "sparing effects", like the omega-3 (n-3) sparing effect that happens when there are enough monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) in the diet [92]. Diets based on TM were very rich in oleic and linoleic acid [**Table 2c**], so it is possible that the fish used these FAs as preferential sources of energy, while other FAs tended to accumulate in different tissues like muscle and liver. Continuing with this idea, it has been reported that the metabolism of fish is less efficient when processing SFA and MUFA than PUFA [93,94], so it would be reasonable to assume that this slower consumption of FAs would produce a temporary surplus of lipids that could accumulate in tissues like muscle. This would also make sense with the intermediate result in dT, because the TM meal used for this diet was partially defatted, which could had allowed the fish to still use a small amount of insect fat as a preferential source of energy. Cases similar to this one have already being described, where the addition of alternative and different sources of fat led to its accumulation in organs like muscle or liver, or an increase in whole body fat [95-97].

Table 3. Growth performance, protein utilization, and biometric indices in rainbow trout fed experimental diets.

Growth performance	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
IBW (g)	46.11	46.25	46.08	46.2	46.05	0.11
Initial length (cm)	15.93	16	15.88	15.95	15.95	0.05
FBW (g)	417.13	413.9	417.38	417.72	411.09	3.49
Final length (cm)	29.94	29.86	30.06	30.01	29.84	0.12
SGR (%/day)	2.47	2.46	2.48	2.47	2.46	0.01
DFI (g/100g fish·day)	1.5	1.48	1.49	1.48	1.48	0.01
FCR	0.86	0.85	0.86	0.85	0.85	0.005
Protein utilization	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
PER	2.63^{ab}	2.68^{a}	2.6^{b}	2.64^{ab}	2.67^{a}	0.02
PPV (%)	46.89	48.83	46.66	47.05	47.1	0.68
ADC_{prot} (%)	88.31^{b}	88.86^{b}	88.18^{b}	90.5^{a}	91.1^{a}	0.22
Biometric indices	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
CF (g/cm^3)	1.55	1.55	1.54	1.55	1.55	0.02
HSI (%)	1.33	1.3	1.2	1.17	1.14	0.15
VSI (%)	11.14	10.45	10.02	10.13	9.94	0.36
ISI (%)	4.58^{a}	4.3^{abc}	4.33^{ab}	3.82^{c}	3.99^{bc}	0.11
IL/FL (%)	70.28	66.07	67.16	65	64.21	1.69

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. IBW: initial body weight; FBW: final body weight; SGR (specific growth rate) = [(ln FBW-ln IBW)/days] · 100; DFI (daily feed intake) = [daily feed consumption (g)/biomass (g) at time] · 100; FCR (feed conversion ratio) = [total feed intake (g) / (FBW-IBW)]; PER (protein efficiency ratio) = [total weight gain (g)/protein intake (g)]; PPV (productive protein value) = [(protein gain (g) / protein intake (g)) · 100]; ADC_{prot} (apparent digestibility coefficient of the protein) = [1-(marker in diet/marker in faeces) · (% protein in faeces/ % protein in diet)] · 100; CF (condition factor) = [weight (g)/length^3 (cm)] · 100; HSI (hepatosomatic index) = [wet liver weight/FBW] · 100; VSI (viscerosomatic index) = [wet visceral weight/FBW] · 100; ISI (intestinal somatic index) = [wet intestine weight/FBW] · 100; IL/FL (intestine length/fish length) = [intestine length/fish length] · 100. $^{a, b, c}$ show statistically significant differences among diets ($p < 0.05$); values expressed as mean ± standard error of the mean (SEM; n=4 tank per diet).

The FA profile of the fillets [**Table 6**] underwent changes that, mostly, agreed with the composition of the diets [**Table 2c**]. The proportion of SFA (myristic, palmitic and stearic) was reduced in most experimental diets. Diets rich on insect fat went up on oleic (T diet) and linoleic (T, TO1 and TO2) acids. Some among the main long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), suffered decreases in T diet, while this decrease did not happen for the case of dT, TO1 and TO2. It is known that the fat of terrestrial insects has a very different FA composition than that of fish, especially regarding MUFA like oleic acid, and LC n-3 PUFA like EPA and DHA [11], so it is not surprising that T diet had the highest levels of oleic acid and the lowest levels of LC n-3 PUFA. Actually, this is a point strongly supported by several studies [16-20], and is possibly the first nutritional drawback of using insects as ingredients for fish. Two strategies were followed in the present experiment to solve this problem: lowering the

contribution of insects to the overall fat of the diet with a partially defatted IM (dT diet), and supplementing other diets with an experimental n-3 rich oil (EO; diets TO1 and TO2). In all three cases, indeed, the problem was solved; however, it is interesting to notice that, in some cases, this problem was not only solved but overcompensated, such as the case of an increase in EPA and DPA in treatments TO1 and TO2, when comparing with C. An interesting research by Turchini *et al*. [92] described that the metabolism of murray cod (*Maccullochella peelii*) has an n-3 sparing effect mechanism, which tends to prioritise particular FAs for energy production, especially MUFA and, to a lesser extent, SFA. TO1 and TO2 were diets with more diverse sources of fat than the other three, so it is possible that this n-3 sparing effect made the production of energy more efficient, allowing a surplus of LC n-3 PUFA (EPA and DPA in this case) to accumulate in other tissues like muscle. This same effect was partially highlighted in dT diet as well, showing higher levels of eicosatetraenoic acid and DPA than C diet. Due to the fact that the defatting process of the IM was not absolute, it is possible that the small amount of TM fat within the defatted IM (8.9 %, around 1.6 % of total feed) allowed again the use of insect-sourced MUFA to promote a partial sparing of the LC n-3 PUFA, making sense with the previously mentioned. Basing ourselves on the current bibliography [96], another analysis was done in order to support the previously mentioned. The proportion of FAs between the different experimental treatments and C was calculated, both for diets and fish fillets, and expressed in the form of graphics [**Figure 1**]. Comparing these proportions and values on a feed-to-fillet basis, the data gives an interesting representation on how the physiology of the fish may be saving and accumulating n-3, while the amount of other FAs, especially MUFA, becomes proportionally lower.

Table 4. Effect of dietary treatments on butchering yield of fish

Butchering yield	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
Dressing yield (%)	89.95	90.6	90.54	90.87	91.28	0.32
Filleting yield (%)	57.91	58.73	59.5	60.3	60.16	0.59

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. Dressing yield = (wet gutted body weight/FBW) · 100; Filleting yield = (wet fillet weight/FBW) · 100. Values are expressed as mean ± standard error of the mean (SEM; n=4 tank per diet).

Table 5. Effect of dietary treatments on proximate composition of rainbow trout raw fillets

Proximate composition (wet basis)	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
Moisture (%)	73.55^{a}	71.86^{b}	72.95^{ab}	72.53^{ab}	73.03^{ab}	0.31
Protein (%)	20.29	20.69	20.39	20.28	20.11	0.27
Fat (%)	1.14^{b}	3.06^{a}	1.88^{ab}	3.16^{a}	2.27^{ab}	0.41
Ash (%)	1.3	1.32	1.31	1.33	1.31	0.02
Calcium (mg/kg)	182	162.13	164.63	147.25	126.38	12.41
Phosphorus (g/kg)	2.58	2.6	2.53	2.62	2.45	0.02

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. $^{a, b, c}$ *show statistically significant differences among diets ($p < 0.05$); values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet).*

Another important feature during the evaluation of the final product is its perceived quality. In this experiment, the texture and colour of fillets were analysed objectively through instrumental methods, but also subjectively through direct sensorial methods. For the case of direct sensorial analyses, both raw and cooked fillets were evaluated.

Table 6. Effect of dietary treatments on fatty acids composition of rainbow trout fillets

Fatty acids profile of fillets (% of total FA)	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
Myristic (MYR; C14:0)	3.06^{a}	2.84^{ab}	2.36^{c}	2.6^{bc}	2.45^{c}	0.07
Palmitic (PA; C16:0)	23.2^{a}	20.09^{bc}	18.97^{c}	19.59^{c}	21.08^{b}	0.28
Palmitoleic (POA; C16:1n-7)	4.36^{a}	3.48^{b}	4.18^{a}	2.93^{c}	2.26^{d}	0.12
Stearic (STE; C18:0)	6.28^{a}	5.05^{c}	5.53^{b}	4.37^{d}	3.92^{e}	0.09
Oleic (OA; C18:1n-9)	19.54^{bc}	25.16^{a}	19.28^{bc}	21.15^{b}	18.6^{c}	0.46
Vaccenic (VA; C18:1n-7)	2.89^{a}	1.87^{c}	2.09^{b}	1.35^{d}	0.99^{e}	0.03
Linoleic (LA; C18:2n-6)	7.13^{e}	14.19^{a}	9.57^{d}	13.36^{b}	12.54^{c}	0.14
Linolenic (ALA; C18:3n-3)	0.97^{ab}	0.99^{a}	0.92^{bc}	0.88^{c}	0.78^{d}	0.01
Stearidonic (SDA; C18:4n-3)	0.31	0.24	0.57	0.5	0.54	0.08
Eicosenoic (GOA; C20:1n-9)	1.48^{a}	1.14^{b}	0.75^{c}	0.74^{c}	0.63^{c}	0.06
Arachidonic (ARA; C20:4n-6)	1.29^{a}	1.05^{b}	1.34^{a}	1.14^{ab}	1.17^{ab}	0.05
Eicosatetraenoic (ETA; C20:4n-3)	0.29^{b}	0^{c}	0.62^{a}	0.41^{ab}	0.36^{b}	0.05
Eicosapentaenoic (EPA; C20:5n-3)	3.29^{c}	2.34^{d}	3.83^{bc}	4.09^{b}	5.33^{a}	0.15
Docosapentaenoic (DPA; C22:5n-3)	1.13^{c}	0.86^{d}	1.29^{b}	1.31^{b}	1.69^{a}	0.03
Docosahexaenoic (DHA; C22:6n-3)	18.95^{a}	15.22^{b}	18.86^{a}	17.51^{ab}	19.94^{a}	0.69
Other (up to 100 %)	5.82	5.47	9.84	8.07	7.72	0.43
∑Saturated fatty acids (SFA)	32.53^{a}	27.98^{b}	26.85^{b}	26.56^{b}	27.44^{b}	0.4
∑Monounsaturated fatty acids (MUFA)	28.28^{b}	31.65^{a}	26.31^{b}	26.16^{b}	22.47^{c}	0.62
∑Polyunsaturated fatty acids (PUFA)	33.38^{c}	34.9^{c}	37^{bc}	39.21^{ab}	42.36^{a}	0.91
∑Omega-3 (n-3)	24.95^{b}	19.65^{c}	26.08^{ab}	24.71^{b}	28.65^{a}	0.85
∑Omega-6 (n-6)	8.42^{e}	15.24^{a}	10.91^{d}	14.5^{b}	13.71^{c}	0.15
∑Omega-6/∑Omega-3 (n-6/n-3)	0.34^{d}	0.78^{a}	0.42^{cd}	0.59^{b}	0.48^{c}	0.02
Peroxidability index (PI)	195.51^{b}	163.09^{c}	203.75^{ab}	196.46^{ab}	224.63^{a}	6.5

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. $^{a, b, c, d, e}$ *show statistically significant differences among diets ($p < 0.05$); values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet).*

Talking about the instrumental analyses, no differences were found among treatments for texture [**Table 7**]. Colour analyses revealed a higher level of lightness (L) in TO1 than in T, and also a lower level of redness (a*) in TO2 than in C diet [**Table 8**]. The entire set of direct sensorial analyses only highlighted a higher level of brightness in the general appearance of cooked fillets for C and T diets than the case of TO2 diet.

Put together, these analyses aim to the idea that the tested ingredients did not alter the organoleptic traits of the fish in an important way. However, the lower level of redness in TO2 of raw fillets suggests that the EO could provoke a more greenish shading colour in these fillets. It is interesting to notice that raw, objective lightness, and cooked, subjective brightness, which

should be similar traits, offered results very close to opposite, suggesting that the effects of the experimental ingredients (IMs and EO), which should be in line with the different nature of the fat, were different before and after the cooking process. There are not many insect-fed fish studies in which the organoleptic profile was evaluated. However, some of them agree with the present results, since it is not unusual to see changes in flesh and/or skin colour [88,99,100], while differences in other traits are less common [101].

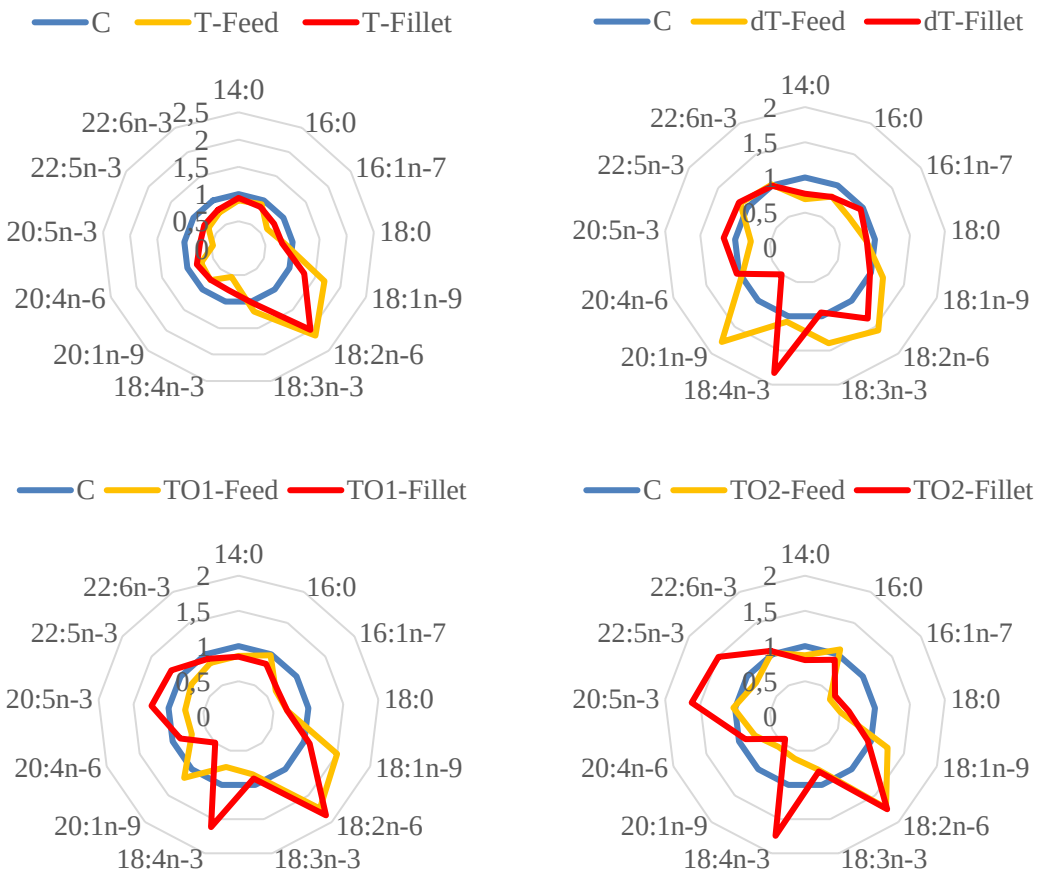

Figure 1. *Comparison between feed and fillet FA profiles, on an experimental treatment vs. control treatment (C) basis, using C diet as a normality baseline with a value of 1. C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched oil (EO); TO2: T diet supplemented with 7.24 % of EO.*

3.3. Liver performance and antioxidant status

There were no changes from the point of view of liver intermediary metabolism [**Table 11**], while liver histomorphology only showed minor differences for hepatocyte lipidic accumulation, being higher on dT, TO1 and TO2 treatments [**Table 12**]. Liver antioxidant status showed lower levels of glutathione peroxidase (GPx) in T, TO1 and TO2, and higher levels of malondialdehyde (MDA) in TO1 and TO2 [**Table 13**].

Liver function remained mostly unaffected among diets, which is often described in insect-fed studies that measured similar enzymatic and histomorphological variables in salmonids [15,34,102,103]. However, the few changes highlighted in the current study can be explained establishing a relationship between liver lipid accumulation and antioxidant status. As previously

mentioned about fillet FA composition, it is possible that the higher availability of MUFA and linoleic acid in insect-based diets, together with an n-3 sparing effect [92] allowed insect-fed fish to have a surplus of these n-3. Following this line of thought, this was reflected in the higher level of lipid accumulation in three out of four insect-based diets (dT, TO1 and TO2). Furthermore, if livers followed the performance of fillets, this would had led to the higher level of MDA in liver that was described as well, because LC n-3 PUFA are characterised for having higher peroxidability indices than other FAs [54,104], which was reflected in the results of this work [**Table 6**]. In this way, and even though IMs are believed to have a positive effect enhancing the antioxidant response [13,14,105-107], the levels of GPx went down in T, TO1 and TO2, probably as a consequence of a long-term depletion of this molecule. Perhaps, the level of antioxidants in the experimental diets was suboptimal, because another experiment with gilthead seabream (*Sparus aurata*) under similar conditions described different results [29]. As such, it is possible that a special addition of antioxidants would had prevented this effect in TO1 and TO2. However, because growth and other relevant variables related to animal welfare were unaffected, it is easy to assume that even these different levels of MDA and GPx can be considered within sustainable, healthy limits, at least for the length of the experiment (89 days).

Table 7. Effect of dietary treatments on the texture of raw rainbow trout fillets (instrumental analysis)

Texture parameters in raw fillets	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
Brittleness	64.13	58.76	71.32	78.6	61.16	7.26
Hardness	182.34	144.48	156.93	179.01	121.7	23.49
Cohesiveness	0.44	0.49	0.42	0.4	0.45	0.03
Adhesiveness	-3.78	-3.88	-3.01	-2.82	-3.96	0.36
Elasticity	1	1	1.01	1.02	1.01	0.01
Chewiness	79.65	72.47	66.74	73.7	54.25	11.93
Gumminess	79.58	72.37	66.3	72.46	53.73	11.96

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. Values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet).

Table 8. Effect of dietary treatments on the colour of raw rainbow trout fillets (instrumental analysis)

Colour parameters in raw fillets	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
Lightness (L)	58.08^{ab}	57.16^{b}	59.05^{ab}	59.55^{a}	58.95^{ab}	0.54
Redness (a*)	-1.57^{a}	-1.74^{ab}	-1.80^{ab}	-1.74^{ab}	-2.08^{b}	0.09
Yellowness (b*)	7.64	6.48	5.84	6.9	7.05	0.52
Chroma (C^*_{ab})	7.83	6.74	6.13	7.13	7.38	0.48
Hue (H^o_{ab})	102.91	106.04	107.82	104.35	106.14	1.68

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. $^{a, b}$ show statistically significant differences among diets ($p < 0.05$); values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet).

3.4. Immunological system and plasmatic metabolites

Few differences were found after the analyses of skin mucus and plasma parameters related to the immune system, as well as the levels of plasmatic glucose and triglycerides [**Table 14**]. There were no statistically significant differences for any of the variables measured in plasma. In skin mucus, acid phosphatase showed lower values in C, T and dT treatments, and a higher one for TO1, with a similar trend in alkaline phosphatase, but showing significant differences only between C and TO1.

Table 9. Effect of dietary treatments on whole fish and fillets of raw rainbow trout (sensorial analysis)

Attributes in raw fish	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
Acceptability	2.87	2.7	2.78	1.97	2.4	0.19
Colour	5.37	5.09	5.5	5.99	5.48	0.27
Texture	2.74	1.98	2.16	1.87	1.85	0.2
Odour	2.75	2.68	2.55	2.67	2.7	0.43
Quality Index (QI)	3.12	3.77	4	3.85	4.69	0.42

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. Values are expressed as mean ± standard error of the mean (SEM; n=13 panellists per diet).

Similar to the case of the antioxidant response, it is often described that IMs might have a positive effect over the performance of the immune system. Henry *et al*. [13] described an enhancement in the trypsin inhibition, bacteriolytic and myeloperoxidase activities of rainbow trout serum after the partial substitution of FM with TM meal. Kumar *et al*. [15] highlighted an increased lysozyme activity in rainbow trout serum after a partial substitution of FM with black soldier fly (*Hermetia illucens*) meal, but also an increased peroxidase activity after a total replacement of fish oil with black soldier fly oil. Interestingly, two of our own past experiments highlighted opposite results to the ones described in this manuscript, with a lower level of alkaline phosphatase for three out of four insect-based diets in one of those experiments [18], and a lower level of acid phosphatase in a diet based on TM in the other one [34]. However, these opposite results were found in different tissues, the present case being one where the highest values of phosphatases were found in skin mucus and not in plasma. Phosphatases are not only enzymes related to external stressors and infections [108-110], but also good indicators of tissue damage [111,112]. TO1 was one of the diets with the highest levels of liver MDA and lipidic accumulation, and also had some of the highest values of LC n-3 PUFA in fillet (DPA and EPA). Considering that fish skin is an important reservoir of LC n-3 PUFA [113], it is no surprise that skin mucus, a tissue that is persistently exposed to external aggressions, showed a higher expression of these enzymes in TO1, the case of TO2 being closely behind. There is not a clear evidence able to justify why these changes in the immune system occur after the inclusion of IMs in the feed, but it is often theorised that different insect related components such as chitin, certain antibacterial peptides, or the lauric acid of black soldier fly could be, at least, partially responsible for these effects [14,114-116]. Plasmatic glucose levels and triglycerides remained unaffected,

which are a good sign that fish physiology could compensate what provoked the changes in liver MDA and skin mucus phosphatases.

Table 10. Effect of dietary treatments on cooked rainbow trout fillets (sensorial analysis)

Attributes in cooked fillets	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
Appearance						
Brightness	9.04[a]	9.03[a]	8.68[ab]	8.93[ab]	7.27[b]	0.38
Exudate colour	7.48	7.53	8.06	7.88	8.17	0.66
Colour uniformity	7.84	7.86	6.88	7.06	6.31	0.67
Colour intensity	6.83	7.03	5.18	5.23	3.86	1
Odour intensity	6.58	5.95	5.05	4.7	5.88	0.53
Taste						
Sweet	2.78	1.56	1.17	2.58	1.29	0.61
Salty	2.12	2.08	2.11	1.81	2.65	0.35
Bitter	0.73	1.44	1.5	1.11	1.69	0.43
Acid	0.4	0.49	0.92	0.64	0.6	0.35
Texture						
Hardness	2.82	2.4	3.4	2.01	3.28	0.61
Juiciness	5.59	4.9	4.1	6.22	3.93	0.61
Fatty	2.24	3.28	2.75	2.6	2.15	0.48
Elastic/gumminess	4.14	3.22	4.8	3.57	3.77	0.57
Teeth adherence	3.82	2.94	4.56	3.82	4.23	0.55
Flavor						
Rancid	1.18	1.35	1.22	1.05	1.64	0.31
Vegetable	1.57	0.93	0.99	0.88	0.89	0.23
Mud/earthy	3.28	3.66	2.92	2.58	3.24	0.42
Sea	3.07	2.82	2.43	2.7	3.18	0.35
Fatty sensation	1.7	2.01	2.04	2.15	1.44	0.47

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. [a, b] show statistically significant differences among diets ($p < 0.05$); values are expressed as mean ± standard error of the mean (SEM; n=8 panellists per diet).

Table 11. Effect of dietary treatments on liver intermediary metabolism enzymes of rainbow trout

Liver intermediary metabolism enzymes	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
FBP_{ase} (mU/mg prot.)	80.2	85.22	62.57	62.41	83.08	6.6
PK (mU/mg prot.)	399.91	740.88	913.83	817.13	774.73	115.64
GPT (mU/mg prot.)	539.02	556.58	486.27	529.78	534.36	57.53
GOT (mU/mg prot.)	3,310.83	3,049.77	2,731.5	2,377.5	2,391.62	267.35
GDH (mU/mg prot.)	851.22	738.61	652.25	683.12	758.58	60.79

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. FBP_{ase}: fructose 1,6-bisphosphatase; PK: pyruvate kinase; GPT: glutamate pyruvate transaminase; GOT: glutamate oxaloacetate transaminase; GDH: glutamate dehydrogenase. Values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet).

Table 12. Effect of dietary treatments on liver histomorphology of rainbow trout

Liver histomorphology (μm)	C	T	dT	TO1	TO2	SEM
Hepatocyte nucleus width	2.05	2.04	2.01	2.09	2.1	0.04
Hepatocyte cytoplasm width	4.45	4.42	4.51	4.59	4.62	0.05
Lipid accumulation in hepatocytes	+	+	++	++	++	
Presence of necrotic focuses	-	-	-	-	-	

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. Values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet).

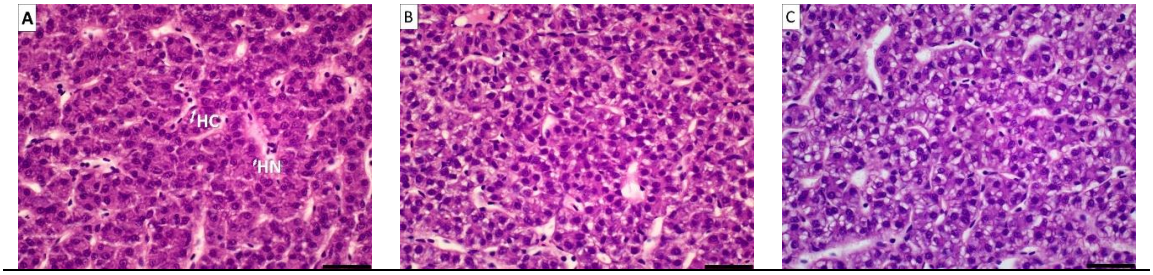

Figure 2. *Visual example of the degree of lipid accumulation in hepatocytes. A: almost no lipid accumulation (-); B: minor degree of lipid accumulation (+); C: medium degree of lipid accumulation (++). Picture A also shows an example of quantitative measures (HC: hepatocyte cytoplasm; HN: hepatocyte nucleus).*

Table 13. Effect of dietary treatments on liver antioxidant status of rainbow trout

Antioxidant enzymes and lipid peroxidation	C	T	dT	TO1	TO2	SEM
SOD (U/mg prot.)	282.36	271.14	304.95	299.62	277.21	10.42
CAT (U/mg prot.)	325.45	342.72	374.34	378.66	368.94	18.48
GPx (mU/g tissue)	16.53^{a}	13.1^{b}	14.03^{ab}	10.41^{c}	10.26^{c}	0.62
GR (mU/mg prot.)	7.73	6.3	6.82	6.29	6.65	0.56
G6PDH (mU/mg prot.)	57.74	50.21	47.67	44.28	44.08	7.31
MDA (nmol/g tissue)	65.06^{b}	65.06^{b}	89.58^{ab}	99.54^{a}	111.32^{a}	7.12

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; G6PDH: glucose-6-phosphate dehydrogenase; MDA: malondialdehyde. $^{a, b, c}$ *show statistically significant differences among diets ($p < 0.05$); values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet).*

3.5. Gut health

Gut health was analysed from two different approaches: histomorphology (distal intestine and pyloric caeca), and microbiota study.

3.5.1. Intestinal histomorphology

Concerning the status of intestinal histomorphology, no changes were highlighted for any of the quantitative variables, neither in distal intestine, nor in pyloric caeca. Minor changes are described for the qualitative analysis: in distal intestine, the level of loss of enterocyte vacuoles was slightly higher in C (+) than in the rest of the diets (-); in pyloric caeca, the level of inflammatory infiltration in submucosa and lamina propria layers was slightly higher in C and TO1 (+) than in T, dT and TO2 (-). Both qualitative variables (inflammatory infiltration and loss of enterocyte vacuoles) are signs of an abnormal immunological status of the gastrointestinal tract

(GIT), so it is reasonable to assume that these results were related to the same cause. It is known that several vegetable ingredients like soybean meal can cause, among others, undesired effects in fish GIT such as the previously mentioned [3,117-119], but it has also been described that IMs or even insect oil can provoke a reduction of these inflammatory effects [15,120]. Even though the present experiment did not reveal a severe case of inflammation, it is interesting to notice that three out of four insect-based diets (T, TO1 and TO2) had a slightly higher amount of vegetable ingredients than C, suggesting that this inhibitory effect might be considerable. However, the different nature of the fat can influence the level of enterocyte vacuolization [121], so this could had been another minor factor involved in this change. The rest of the variables remained very stable among all treatments, which in general follows the trend of other studies related to insect-sourced ingredients as the main target, especially regarding TM [24,122,123].

Table 14. Effect of dietary treatments on immunological status (plasma and skin mucus) and plasma metabolites of rainbow trout

Immunological system and metabolites (plasma)	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
Lysozyme (U/mL)	2.22	2.22	1.99	1.68	2.62	0.21
Antiprotease (U/mg prot.)	163.75	168.86	164.46	164.12	130.77	9.21
Acid phosphatase (mU/mg prot.)	973.55	919.88	746.49	1,019.55	926.23	80.57
Alkaline phosphatase (mU/mg prot.)	1,040.85	834.1	1,054.88	1,036.89	930.7	63.17
Peroxidase (U/mg prot.)	0.82	0.94	0.76	0.81	0.76	0.07
Immunoglobulins (mg/mL)	17.48	18.18	16.19	15.1	16.25	0.86
Glucose (nmol/L)	3.77	3.69	3.63	3.76	3.71	0.13
Triglycerides (nmol/L)	4.9	5.81	4.87	5.86	5.61	0.81
Immunological system (skin mucus)	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
Acid phosphatase (mU/mg prot.)	1,256.02^{b}	1,095.21^{b}	1,376.64^{b}	2,471.43^{a}	1,642.04^{ab}	207.11
Alkaline phosphatase (mU/mg prot.)	1,286.65^{b}	2,196.06^{ab}	2,781.82^{ab}	3,320.95^{a}	2,694.87^{ab}	431.58
Peroxidase (U/mg prot.)	16.83	23.09	13.85	20.41	11.52	3.49
Esterase (U/mg prot.)	11.91	13.03	9.67	11.7	9.44	1.24
Carbonic anhydrase (mU/mg prot.)	685.83	485.73	429.89	131.3	460.53	153.97

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. HEWL: Hen Egg White Lysozyme. $^{a, b}$ show statistically significant differences among diets ($p < 0.05$); values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet).

3.5.2. Digesta microbiota analyses

Alpha diversity

C diet had the highest score for Chao1 index, followed by T and dT, and with the lowest values for TO1 and TO2 diets. No differences were highlighted for Simpson index. Shannon index was higher in T than in dT [**Table 15**].

Put together, the experimental ingredients (IMs, and especially the defatted one and EO) reduced the amount of absolute microbial populations. Chao1 index (richness) was significantly

lowered by dT, TO1 and TO2, which aims to the idea that the inclusion of IMs should be related to this. Other experiments with both whole [124] and partially defatted [125] IMs got similar

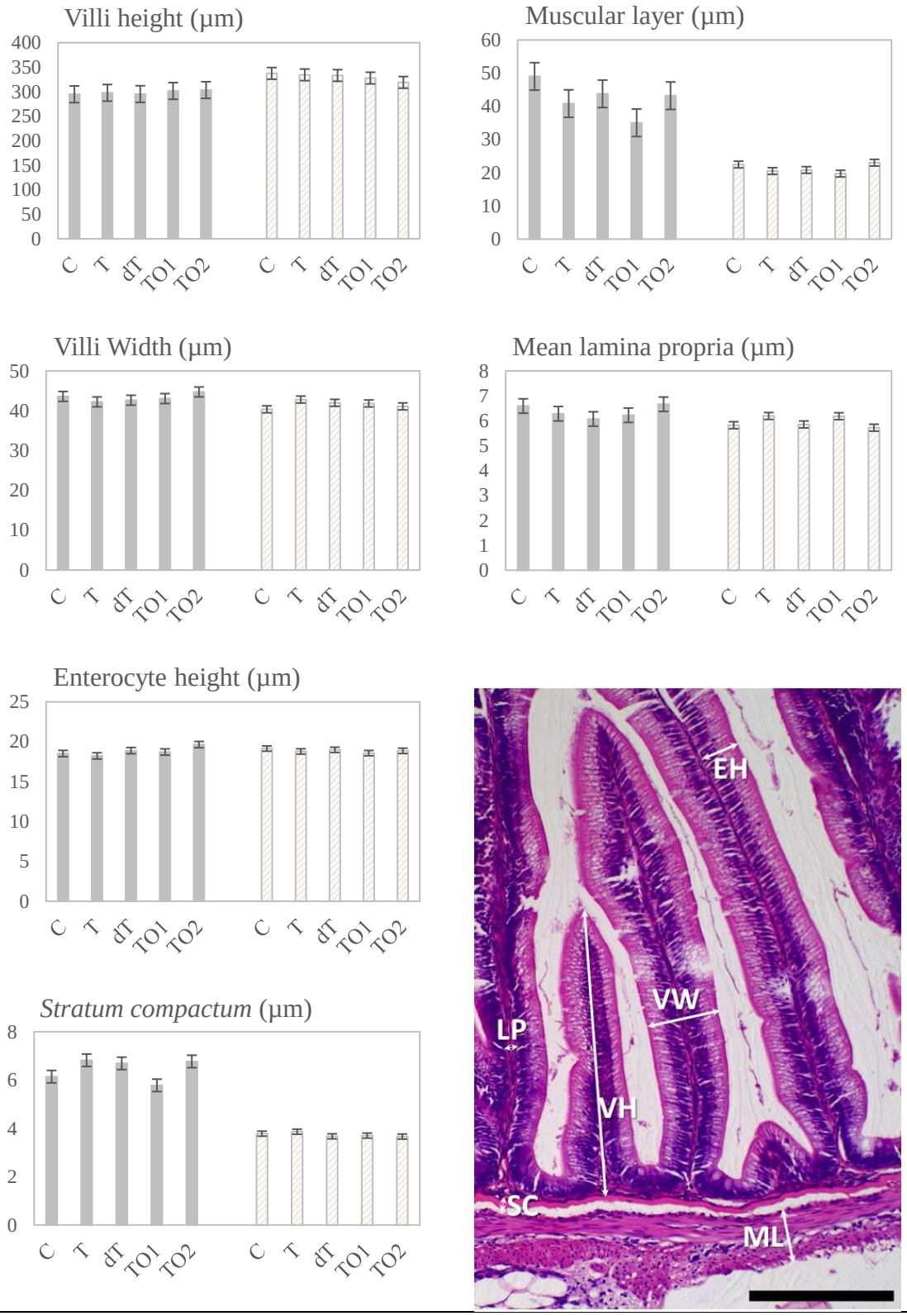

Figure 3. *Quantitative measures during histomorphology analyses of rainbow trout gut. C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. Grey bars: distal intestine measures; striped bars: pyloric caeca measures. Values expressed as mean ± standard error of the mean (SEM; n = 4 tanks per diet). Microphotograph representative of measures for gut: villi height (VH), villi width (VW), enterocyte height (EH), stratum compactum (SC), muscular layer width (ML), lamina propria width (LP). Scale bar = 100 µm.*

results, but it would be worthwhile to mention that the opposite case has also been reported [31,126,127]. Shannon index only showed differences between T (higher) and dT (lower), possibly meaning that the evenness of the digesta microbiome was affected by the defatting process of the IM used in dT. This is partially supported by the current bibliography about insect-fed fish, since other trials described lower levels of Shannon index after using partially defatted IMs [32,128], even in the feed itself [129].

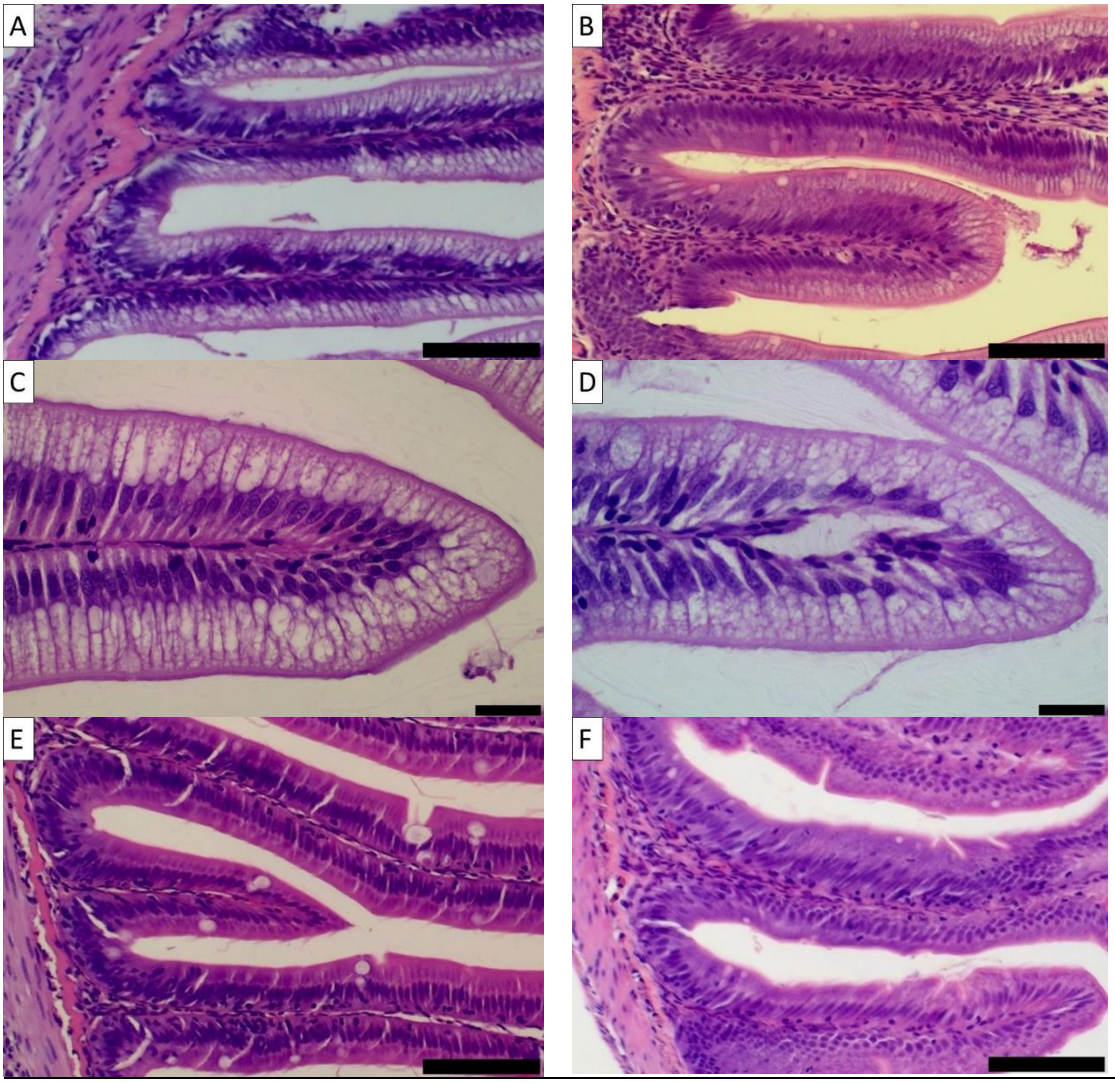

Figure 4. *Visual example of the histomorphology qualitative analyses carried out in rainbow trout intestine. Pictures **A** and **B** (distal intestine), with **E** and **F** (pyloric caeca) are examples of the degree of inflammatory infiltration, and were taken at 100x magnification, scale bars = 50 μm. Pictures **C** and **D** (distal intestine) are examples of the degree of vacuoles loss, and were taken at 400x magnification, scale bars = 10 μm. Pictures on the left represent a negative level (-) of the variables, while pictures on the right represent a low level (+).*

Beta diversity

Results are given at phylum and genus levels, and showed several differences among treatments [**Tables 16 and 17**]. At phylum level, Bacillota was the most dominant population with a total abundance that went from ~65 to ~84 % of the total. C treatment showed the highest values for Actinomycetota, with lower numbers for T, dT and TO2, staying TO1 in the middle. Bacteroidota was also higher for C, followed by T and dT, and with significant differences for the lowest values of TO1 and TO2. Cyanobacteria followed a similar trend, but in this case, dT had

significant differences when compared with C and T, and TO1 offered the lowest results. Bacillota offered opposite results, with a higher value in TO2, middle scores for dT and TO1, and significantly lower levels for T and, finally, C. At genus level, *Peptostreptococcus* (15.84-27.04 %), *Peptoniphilus* (13.06-18.47 %), *Nostoc* (4.49-13.18 %) and *Streptococcus* (7.16-8.75 %) were the most dominant. The number of *Bacteroides* and *Falsiporphyromonas* was higher in C, with a decreasing trend towards T and dT, and significant differences for TO1 and TO2. The amount of *Nostoc* was equivalent in C and T, with middle levels in dT and lower levels for TO2 and TO1 (in that order). *Bacillus*, *Brevibacillus* and *Enterococcus* followed the same trend, with higher scores for T, TO1 and TO2, and lower for C and dT. dT showed the highest numbers for *Helcococcus*, *Peptoniphilus*, *Citroniella* and *Peptostreptococcus*, with medium values in C, TO1 and TO2 diets, and the lowest values in T, the case of *Peptostreptococcus* being more accused in these differences.

Table 15. Effect of dietary treatments on microbiota alpha diversity of rainbow trout distal intestine digesta

Alpha diversity index	C	T	dT	TO1	TO2	SEM
Chao1	309.75^{a}	278.26^{ab}	240.81^{bc}	230.33^{c}	224^{c}	9.68
Simpson	0.93	0.95	0.91	0.93	0.93	0.01
Shannon	3.85^{ab}	4.03^{a}	3.67^{b}	3.78^{ab}	3.79^{ab}	0.07

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. $^{a, b, c}$ show statistically significant differences among diets ($p < 0.05$); values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet).

Table 16. Effect of dietary treatments on OTU composition at phylum level of rainbow trout distal intestine digesta

Relative OTU composition at phylum level	C	T	dT	TO1	TO2	SEM
Actinomycetota[1]	2.76^{a}	1.83^{b}	2.05^{b}	2.21^{ab}	1.78^{b}	0.15
Bacteroidota[2]	12.44^{a}	11.21^{ab}	8.52^{ab}	5.84^{b}	5.50^{b}	1.41
Cyanobacteria	13.86^{a}	12.51^{a}	7.99^{b}	4.75^{c}	6.11^{bc}	0.58
Bacillota[3]	65.67^{c}	69.17^{bc}	78.78^{ab}	79.04^{ab}	83.59^{a}	2.97
Pseudomonadota[4]	4.14	4.08	1.92	7.61	2.01	2.79
Other	1.13	1.20	0.74	0.54	1.00	-

1: phylum Actinomycetota, previously named as Actinobacteria [140]; 2:phylum Bacteroidota, previously named as Bacteroidetes [140]; 3: phylum Bacillota, previously named as Firmicutes [140]; 4: phylum Pseudomonadota, previously named as Proteobacteria [140]. C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. $^{a, b, c}$ show statistically significant differences among diets ($p < 0.05$); values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet). OTU: Operational taxonomic units.

Beta-diversity was also affected by the experimental diets [**Tables 16 and 17**]. At phylum level, there was an accused increase in Bacillota, especially for dT, TO1 and TO2, which seems to be a very common and steady result associated to the increase of IMs in fish feedings [30,31,126,130]. This trend was inversely followed by Bacteroidota a point where the bibliography offers more disperse conclusions, even though a decrease in this population has been described as well [127,131,132].

Table 17. Effect of dietary treatments on OTU composition at genus level of rainbow trout distal intestine digesta

Relative OTU composition at genus level	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
Bacteroides	3.7^{a}	3.61^{ab}	2.59^{abc}	1.69^{bc}	1.42^{c}	0.44
Falsiporphyromonas	7.47^{a}	6.46^{ab}	5.11^{ab}	3.62^{b}	3.55^{b}	0.83
Nostoc	13.18^{a}	11.91^{a}	7.56^{b}	4.49^{c}	5.77^{bc}	0.56
Bacillus	0.21^{b}	2.94^{a}	0.25^{b}	2.79^{a}	2.5^{a}	0.17
Brevibacillus	0.02^{b}	8.06^{a}	0.004^{b}	7.12^{a}	8.05^{a}	0.5
Enterococcus	0.23^{b}	1.78^{a}	0.27^{b}	2.51^{a}	2.41^{a}	0.19
Streptococcus	7.45	7.16	8.75	8.12	7.85	0.47
Helcococcus	1.18^{ab}	0.86^{b}	1.39^{a}	1.22^{a}	1.13^{ab}	0.08
Peptoniphilus	14.86^{ab}	13.06^{b}	18.47^{a}	16.2^{ab}	17.64^{a}	0.9
Citroniella	2.01^{a}	1.55^{b}	2.27^{a}	2.04^{a}	1.95^{ab}	0.1
Peptostreptococcus	20.23^{bc}	15.84^{c}	27.04^{a}	19.33^{bc}	23.05^{ab}	1.55
Other	29.46	26.77	26.3	30.86	24.67	-

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. $^{a, b, c}$ *show statistically significant differences among diets ($p < 0.05$); values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet). OTU: Operational taxonomic units.*

The analysis at genus level was reinforced with principal components analysis (PCA) between microbiota genus *vs*. amino acids (AA) and FA composition of the diets [**Figure 5**]. In this way, three main groups of results are highlighted. First and foremost, the amount of *Bacillus*, *Brevibacillus* and *Enterococcus* suffered drastic changes among diets, with results close to zero in C and dT. According to the PCA, these differences were related to the levels of omega-6 fatty acids (n-6) and linoleic acid in diets. *Bacillus* and *Enterococcus* are known for having probiotic properties in fish and promoting intestinal health [133-135]. Similar results were found in other experiments with insect-fed fish [122,126,130,136] where these or other lactic-acid bacteria proliferated, which is positive from the point of view of using IMs for fish diets. Actually, these results, and especially those of TO1 and TO2, matched the higher ADC_{prot} previously described in this same study [**Table 3**]. Due to the fact that, in our case, C and dT had the lowest values for these bacteria, even though these bacteria populations showed some kind of tropism towards IMs, it is possible that insect fat acted as the most relevant component. The second group of results comprised *Bacteroides*, *Falsiporphyromonas* and *Nostoc*, which suffered a moderate decrease in dT and a more pronounced decreased in TO1 and TO2. These populations showed a strong correlation with the AAs leucine, threonine and aspartate, and with stearic and vaccenic acids, at the same time that they showed an inverse and strong relationship with PUFA. Lastly, *Helcococcus*, *Citroniella*, *Peptostreptococcus* and *Peptoniphilus* conformed a third group that, with due differences, showed intermediate values for C, TO1 and TO2, and opposite behaviour for dT (highest value) and T (lowest value). The PCA revealed a particular relevance of n-3, DHA and EPA for this case, and an interesting interaction with tyrosine, even though its contribution was lesser. According to the composition of the IMs [**Table 1**], the diets [**Table 2b**] and to the bibliography [12], TM is rich in tyrosine, which was reflected on the results of dT but not those of T in the PCA. This aims to the idea that the composition of insect protein and n-3 might be major determinants on the development of these bacteria, while high levels of OA, n-6, or even

the higher n-6/n-3 ratio found in diets with insect fat (particularly marked in T diet) could had acted as inhibitors, which would also make sense with the intermediate levels found in TO1 and TO2. Talking about the particular case of *Peptostreptococcus*, it is an anaerobic bacterium known for its ability to ferment AAs, including among them those with an aromatic group [137,138], tyrosine being one of these AAs. Even though no differences in growth are reported within the present results, *Peptostreptococcus* has also been identified as an indicator taxa of fast-growing rainbow trout [139], which is a positive aspect on the evaluation of defatted TM as an ingredient. Also, the defatting process allows the concentration of more TM protein in a diet formulation and, consequently, a higher amount of tyrosine.

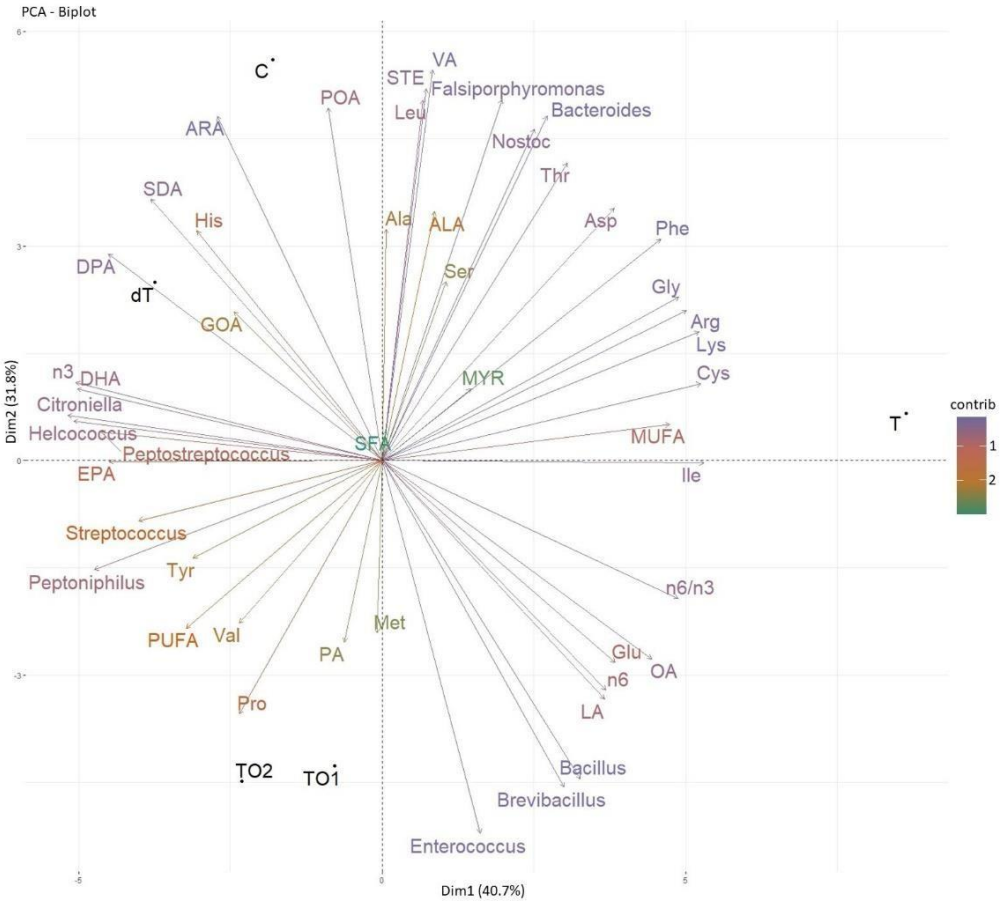

Figure 5. *Principal components analysis (PCA) of main distal intestine digesta microbiota genus of rainbow trout vs. feed composition (amino acids and fatty acids). C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. Acronyms used for amino acids and fatty acids follow the same key as* ***Tables 2b and 2c****.*

4. Conclusions

First and foremost, the proposed diets did not compromise the growth of rainbow trout or the viability of the final product. Also, one of the main objectives of this feeding trial, which was solving the problem with the reduction of long chain omega-3 polyunsaturated fatty acids in fillets of insect-fed fish, was successful. Secondarily, this work also supports some of the points that can be found in the bibliography, like the fact that the nature of the lipids can be decisive due to the

existence of an omega-3 sparing effect in the physiology of rainbow trout, for example, provoking the accumulation of lipids in certain organs like muscle and liver. Also, yellow mealworm meal showed slight but interesting effects on gut health, preventing a slight degree of inflammatory status when compared to C diet, and favouring the promotion of probiotic agents in the microbiome. As a whole, and particularly due to the unexpected performance of the antioxidant response, dT was probably the diet with the most interesting performance. More research is encouraged to support some points that remain unclear, like the relationship between protein digestibility and somatic indexes related to a size reduction of the viscera (hepatosomatic index, viscerosomatic index, intestinal somatic index and filleting yield) and, primarily, the biochemical mechanisms behind the effects of insect meals in general, and chitin in particular, on the immunological and antioxidant response of fish.

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CHAPTER 6 – General discussion

CHAPTER 6. GENERAL DISCUSSION

1. Growth performance, protein use and biometric indices

The review of insect-fed fish studies that was conducted revealed around two hundred research articles that analysed fish growth. Even though this big bank of data has some discrepancies, the most obvious conclusion that can be extracted from it is that, if used within reasonable margins of inclusion in the feed, insects as ingredients for fish do not impair growth in a significant way [1-3]. The results of this thesis were not different. Low and intermediate inclusion levels (5 and 10 %, respectively)[1] of insect meals (IMs) showed a very slight difference in feed conversion ratio between fish fed with black soldier fly (*Hermetia illucens*, HI) and with yellow mealworm (*Tenebrio molitor*, TM). This difference between IMs was more pronounced with the highest inclusion level (18 %)[2]. When the fish were grown to commercial size and fed for longer, the inclusion of TM in the feed maintained good growth results respect to control regardless of whether the IM was defatted or supplemented with omega-3 enriched experimental algal oil (EO).

In general, it could be said that TM is a better alternative for rainbow trout (*Oncorhynchus mykiss*) than HI, but our results, together with what can be found in the bibliography [3-6] prove that HI is neither a bad alternative. It is possible that the composition of HI, slightly different from that of TM in its amino acids (AAs), fatty acids (FAs), and chitin [7,8], makes the inclusion of this insect in feeds for rainbow trout more complicated. These factors could be limiting the maximum amount of this ingredient that can be tolerated by the physiology of rainbow trout. As such, the current results suggest that a theoretical limit could be located somewhere between 10-18 % of HI in rainbow trout feeds, while an 18 % of TM still allowed the development of an optimal growth. This suggests that the proportion of TM in rainbow trout feeds could still be heightened further.

The analyses of protein use revealed very similar results to those of growth, with very small changes among treatments and only one notable exception to this on the highest inclusion level of HI in Study 2. A higher apparent digestibility of the protein (ADC_{prot}) was highlighted for 10 % TM inclusion, meaning that the availability of protein was higher for these fish. It is known that insects are part of the natural diet of rainbow trout [9,10], so it is not strange that the fish are able to digest them efficiently. As for why this did not happen so well in HI diets, it was possibly due to the slightly worse AA profile of HI shown in the studies, together with its higher proportion of chitin, which has been described as a component that can disrupt the digestibility of the protein [11,12]. However, the better ADC_{prot} of TM diet was only 5.2 % higher than control diet, and was not repeated with higher inclusion levels, so it was not given an especial relevance during its interpretation. On the other hand, the lower ADC_{prot} of HI with the highest inclusion of 18 % was substantial (around 12.5 % lower than control diet), and could be one major

[1] Replacement level of fishmeal 15-30 %, respectively.
[2] Replacement level of fishmeal 50 %.

cause of the lower growth reflected in this treatment. In addition to this, Study 3 showed unexpected results with a slightly higher ADC_{prot} for diets enriched with EO. An obvious cause was not found for this change, but it is possible that the changes within intestinal microbiota (described later) could be related to this.

Biometric indices remained very stable during all studies, but dropped some interesting coincidences with other variables. The 10 % TM inclusion showed a lower viscerosomatic index (VSI) than the rest of the treatments during Study 1. The same happened with the intestinal somatic index (ISI) of diets enriched with EO during Study 3. Even though there were no statistically significant differences in Study 2, the VSI showed by the highest HI inclusion had the highest value. These numbers were inversely related with ADC_{prot} in all cases (higher ADC_{prot}, lower VSI and ISI), and the same hypothesis was offered to explain it. It is known that, from the point of view of evolution, animals that base their nutrition on ingredients with lower digestibilities (typically, herbivorous), tend to develop longer intestines [13,14]. It would not be surprising if animals in general, and fish in particular, could have a natural mechanism to adapt the intestinal length to the digestibility of the diet. However, some studies measured this kind of parameters during other trials with insect-fed fish, showing little or no differences [15,16], and the results of this thesis are only partial because no measures were taken to calculate true intestine indices in the first two studies. As such, this will remain as a hypothesis in order to encourage further research. Lastly, when fish were grown to a commercial size, dressing yield and filleting yield did not reveal any statistically significant changes during Study 3.

2. Digestive enzymes

Digestive enzymes were measured in the first two studies, and different trends were found. First, the activity of acidic proteases remained unchanged. Second, alkaline proteases had a stronger activity in diets with IMs, and especially on those with TM. This suggests that IMs suffer a more active digestion during their transit through the intestine. Furthermore, these results show a coincidence with those of ADC_{prot}, with better ADC_{prot} for TM diets, which makes sense with the higher levels of proteases. Finally, amylase showed opposite trends between these two studies. Even though the justification for these results made sense within their contexts, it is complicated to establish a clear explanation on why there were such drastic changes between them. One feasible explanation could be that the inclusion levels of IMs were different, having a difference of almost a 10 % in the total formulation (5-10 % inclusion in Study 1 *vs*. 18 % inclusion in Study 2), which could had modified the relevance of the different factors involved. Another possibility would be that the fish of these studies had very different sizes. Study 1 was carried out from ~55.3 g to ~140.5 g, while the second one took place from ~14.6 g to ~75.9 g. It is known that the size of the fish can be very important when it comes to evaluating the digestive function [17-19], so it is possible that diets which seem to be very similar could show very different results for the same parameter if fish have different ages. There are few insect-fed fish studies in which changes in intestinal amylase activity were reported. Two among them described an increase in its activity after the usage of IMs [5,20,21], other four, a decrease [22-24], and another one reported a change related to the portion of the intestine, instead of the use of

IM itself [25]. As such, it is uneasy to establish a direct relationship between amylase and IMs. It is possible that these changes are more conditioned by other factors, like the already mentioned age of the fish, or the proportion of vegetable-sourced ingredients that appear in most experimental diets.

3. Intestine health: histology and microbiota

3.1. Intestine histomorphology

Intestinal histomorphology analyses were carried out during Studies 2 and 3, for distal intestine and pyloric caeca, and from quantitative and qualitative perspectives. Even though many differences were not described among treatments, the results were solid and consistent.

With the highest inclusion levels of IM in the diet (18 %), there was a statistically significant difference in villi height between HI and TM treatments, both for distal intestine and pyloric caeca. This finding is directly related to the lower ADC_{prot} showed by HI, which had a consequential lower growth as well. There were no significant differences in this parameter between control diet and the experimental treatments, which was repeated in Study 3, and which seems to match part of the bibliography. Even though there is some disperse data on the matter, many insect-fed fish manuscripts described little or no changes for the histology of intestinal parameters [26-30]. Other experiments described lower villi heights for HI-based diets [5,31-33], more frequently than for TM [34]. Another parameter that could be related to the lower ADC_{prot} shown by the HI treatment was the lower width of the circular muscular layer. As mentioned in Study 2, a change in muscular layer function could modify indirectly the digestibility of the nutrients by altering the intestinal microbiota [35]. Even though no assumptions can be done at this point, it is possible that a modification of the microbiota could had contributed as well for the decrease of ADC_{prot} and/or the shortening of villi height.

Excluding the previously mentioned, the rest of the variables fell into one of the following categories: either they did not show any changes, or they were related to the inflammatory status of the intestine. For this last case, enterocyte height was higher with TM than in control diet during Study 2, which can be related to the level of enterocyte vacuolisation [36,37]. Indeed, the density of enterocyte vacuoles of control treatment was lower in the mucosa of distal intestine, which was followed by a higher level of inflammatory infiltration in the lamina propria. When the fish were fed for a longer period of time during Study 3, control treatment also revealed a slightly lower level of vacuolisation in the enterocytes of distal intestine, as well as a slightly higher level of inflammatory infiltration in the pyloric caeca. Put together, all these results aim to the idea that treatments without IMs had a discreet but persistent level of inflammation, while IMs tended to soften this effect. Vegetable ingredients are known for provoking effects like these in fish intestine [38-40], while insect-sourced ingredients are known for being able of attenuating them [41,42]. This could be due to one or several among the components of IMs, such as chitin [26] or insect fat [43].

3.2. Microbiota analyses

The microbiota of distal intestine was analysed only during Study 3, where fish reached a higher size and were fed for longer. However, the bibliographic review that was carried out during the elaboration of this thesis allows to expand further what was described in Study 3.

3.2.1. Alpha diversity

Microbiota analyses executed during insect-fed fish experiments drop very different results considering the alpha diversity. As an example of this, there are studies that described an increase in different alpha diversity indices such as the ones shown in Study 3: Chao1 [44,45], Simpson [44,46] and Shannon indices [44,47-49]. There are also cases in which the opposite was reported for the same indices [46,50,51]. These apparently contradictory results could have their fundaments in the nutritional needs of the microbiota or the intestinal dynamics that occur inside the fish, but there might be other factors involved that are virtually impossible to keep under control. During Study 3, the two IMs that were used (full fat and defatted) came from different suppliers, so it would not be a surprise if these two ingredients had different bacterial populations even before the feeds were manufactured. This should give the idea that, when talking about microbiota, the composition of the ingredients used could be just as important as their origin or even their manufacture conditions, something that has already been reported [52].This observation should serve as encouragement to do more research in the different stages that take place during the manufacture processes and the key ingredients that change among experimental treatments, especially in those experiments that are more focused in microbiota.

3.2.2. Beta diversity

The same that was described previously would apply for beta diversity as well, and a proof of this would be the disperse populations that can be found in different manuscripts, coming all of them from different facilities, ingredients and, sometimes, even processes [21,34,53]. If we couple this idea with the fact that microbiota studies are still at a very early stage, it is no surprise that finding some kind of "universal" and sufficiently repeatable trend as a consequence of using IMs in fish feedings is a very harsh task. However, talking about the results on Study 3, and taking aside those populations that are less common in other manuscripts (like *Falsiporphyromonas*, *Nostoc*, *Brevibacillus* and *Peptoniphilus*), there are indeed some trends that are repeated throughout the bibliography. Among them, the ones that seem to be highlighted as most frequent are two: first, that IMs tend to increase the proportion of Bacillota at phylum level [44,45,46,48,51,54]; second, IMs also increased the proportion of lactic-acid bacteria such as *Bacillus* and *Enterococcus* in several cases [26,34,45,50,51,55], which are inside the Bacillota phylum. Bacillota have been associated with chitinolytic activity [56,57] and with a higher absorption of lipids by enterocytes [58], which are both strong advantages from the point of view of using IMs. Lactic-acid bacteria are known for their probiotic properties because they can produce metabolites such as lactic acid, bacteriocins and acetic acid, which potentially contribute to a healthy intestinal epithelium of fish [59,60].

One last and interesting observation was extracted from the bibliography with a brief review through the following procedure. A total of 35 articles were used for the analysis,

considering all those that showed microbiota alpha-diversity results related to an insect-fed fish study. To simplify the interpretation of the data, those manuscript that worked with insects different to HI or TM were left out of the analysis. Also, some experiments for which IM was not considered as their main focus were left out as well, like studies in which the experimental diets with IMs added other experimental ingredients as alternative source of protein in a significant proportion, leaving a total of 26 articles for the analysis. The data was then processed to classify the results as "cases" depending on the IM that was used, as well as its status. For example, if one manuscript described the use of three experimental diets, one with defatted HI, one with full-fat HI, and one with full-fat TM, that manuscript would be considered as three different cases. This classification left a total of 33 cases. Lastly, these cases were evaluated depending on the effect of the IM in the alpha diversity when compared to the control diet that was used, either from the point of view of "positive", "neutral" or "negative" effects on different alpha diversity indices, or the overall interpretation of the case. Among these studies, there are cases in which treatments with HI tended to improve the alpha diversity of the microbiota [**Tables 1 and 2**]. However, cases with defatted HI showed results like this more often (5 cases) [45,47,54,61,62] than cases with full fat HI (4 cases) [26,34,53,54] and what might more important, HI cases with defatted HI showed negative results less frequently (1 case) [63] than cases with full fat HI (3 cases) [29,51,55]. Considering what was previously mentioned about the composition of HI, and particularly its high level of lauric acid, it is possible that this component, known for its antimicrobial properties, could be causing an inhibition of some microbiota populations. Knowing as well that IMs in general have several antimicrobial peptides in their composition [64,65] it would not be strange that with or without the defatting process, insect-based ingredients could cause this same inhibition in different ways. Theoretically, this should also be reflected in the beta diversity of the microbiota as selective inhibition or promotion of different populations. Actually, a similar trend was found as well for TM, with more "negative" cases with full fat TM (3 cases) [21,26,66] than during cases with defatted TM (1 case) [67] [**Tables 1 and 2**]. Knowing that TM does not have a significant proportion of lauric acid in its composition [7,8], it would be reasonable to assume that the defatting process can indeed modify the microbiota, but not only due to the lipid composition of the IM. If we connect this idea with what was previously suggested about the different microbiota found in different experiments, possibly due to the different origin of the ingredients, the big diversity of results in this kind of experiments would make sense.

4. Liver function: intermediary metabolism and liver histology

The liver of rainbow trout was analysed from two points of view: a more functionally oriented approach through the enzymatic activity of intermediary metabolism, and a histomorphological approach to evaluate the general status of this organ.

The hepatic intermediary metabolism remained mostly unaffected during the three studies, especially in the second and third, where no statistically significant changes were reported. The intermediary metabolism can be modified under circumstances where the main components of the diet (protein, lipids and carbohydrates) suffer significant changes [73,74].

Table 1. Contribution of insect meals to alpha diversity

Case	Insect	Type	Alpha diversity effect
Bruni *et al*. 2018 [47]	HI	Defatted	Positive
Antonopoulou *et al*. 2019 [46]	TM	Full fat	Positive
Foysal *et al*. 2019 [68]	HI	Full fat	Neutral
Huyben *et al*. 2019 [54]	HI	Defatted	Positive
Huyben *et al*. 2019 [54]	HI	Full fat	Positive
Józefiak *et al*. 2019a [26]	HI	Full fat	Positive
Józefiak *et al*. 2019a [26]	TM	Full fat	Negative
Jozefiak *et al*. 2019b [34]	HI	Full fat	Positive
Jozefiak *et al*. 2019b [34]	TM	Full fat	Positive
Rimoldi *et al*. 2019 [61]	HI	Defatted	Positive
Terova *et al*. 2019 [44]	HI	Defatted	Neutral
Lu *et al*. 2020 [5]	HI	Defatted	Neutral
Mikoajczak *et al*. 2020 [27]	TM	Full fat	Neutral
Zarantoniello *et al*. 2020 [29]	HI	Full fat	Negative
Fabrikov *et al*. 2021 [21]	HI	Full fat	Neutral
Fabrikov *et al*. 2021 [21]	TM	Full fat	Negative
Jeong *et al*. 2021 [69]	TM	Defatted	Neutral
Panteli *et al*. 2021 [66]	HI	Defatted	Neutral
Panteli *et al*. 2021 [66]	TM	Full fat	Negative
Rimoldi *et al*. 2021 [45]	HI	Defatted	Positive
Terova *et al*. 2021 [67]	TM	Defatted	Negative
Tran *et al*. 2021 [62]	HI	Defatted	Positive
Zarantoniello *et al*. 2021 [70]	HI	Full fat	Neutral
Biasato *et al*. 2022 [50]	HI	Defatted	Neutral
Couto *et al*. 2022 [71]	HI	Defatted	Neutral
Leeper *et al*. 2022 [53]	HI	Full fat	Positive
Pleić *et al*. 2022 [63]	HI	Defatted	Negative
Rangel *et al*. 2022 [57]	HI	Defatted	Neutral
Rangel *et al*. 2022 [57]	TM	Defatted	Neutral
Tran *et al*. 2022 [72]	TM	Defatted	Neutral
Weththasinghe *et al*. 2022 [51]	HI	Defatted	Neutral
Weththasinghe *et al*. 2022 [51]	HI	Full fat	Negative
Yamamoto *et al*. 2022 [55]	HI	Full fat	Negative

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

Table 2. Contribution of insect meals to alpha diversity (data from **Table 1**)

Insect	Type	Positive	Neutral	Negative
HI	Defatted	5	7	1
	Full fat	4	3	3
TM	Defatted	0	3	1
	Full fat	2	1	3

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

Due to the fact that other insect-fed fish trials did not report major differences [21,75-77], it is reasonable that the studies reported in this thesis, which used isoproteic and isolipidic diets, did not perform differently. Nevertheless, there were some minor trends that deserve attention. Talking about the enzymes related to protein metabolism, glutamate dehydrogenase (GDH), glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT), the only one that showed a real change was GOT. Such change occurred between the 10 % inclusion of TM and the inclusion of HI (5 and 10 %) in Study 1, but this trend was almost repeated when the inclusion of IMs reached 18 % during Study 2 (p-value = 0.059), with a minimum in HI and a maximum value in TM. It is not possible to draw a firm conclusion, but maybe the lower ADC_{prot} showed by HI, and particularly by its highest inclusion (18 %), decreased the protein availability in the bloodstream of the fish, which could be related to a lower physiologic activity of this enzyme. Concerning the lipid metabolism, the results on beta-hydroxyacyl-CoA dehydrogenase (HOAD) during Study 1 were contradictory (lowest value in 5 % TM inclusion, highest value in 10 % TM inclusion) and not given a especial consideration. However, the results of Study 3 around the composition of the fillets gave the idea that a metabolic imbalance was taking place after the nature of the dietary fat was modified, but since HOAD and glycerol kinase (GyK) were not measured, it is again not possible to draw a conclusion. Lastly, during Study 1, the 10 % inclusion of IMs showed a difference for citrate synthase (CS) between HI (lowest) and TM (highest). In general, TM diets showed some among the highest numbers for most enzymes related to carbohydrate metabolism, the case of pyruvate kinase (PK) being more consistent (and not statistically significant). If, theoretically, this difference was reflecting something relevant, this could have been due to the slightly higher levels of vegetable ingredients used to balance these diets. It has been described that PK activity and CS gene expression can be induced with these ingredients in fish feeds [78,79].

The liver histology analyses carried out for studies 2 and 3 did not highlight very revealing results, which matches a good part of the bibliography on insect-fed fish [41,70,80]. For the same inclusion level of TM (18 %), a higher accumulation of fat was described for partially defatted TM and diets enriched with EO than for control diet and non-supplemented full fat TM in Study 3, while Study 2 showed no differences at all. The size gap of the fish (~70 g *vs*. ~415 g) and even the short difference in the feeding periods (77 days *vs*. 89 days) are worthy of consideration. In this way, if the fish of Study 2 had reached the size of the fish in Study 3, it is possible that the expected high proportion of monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) in IMs diets would have produced a comparable degree of metabolic imbalance, leading to a similar degree of lipidic accumulation in liver than what was described in Study 3.

5. Animal welfare indicators: antioxidant response, immune system andplasmatic levels

As it was explained during the introduction of this thesis, animal welfare is a topic of growing importance in the eyes of the population, but also from the perspective of professional activities. Taking aside the ethical considerations, which should by themselves be potent motivators to make animal welfare be a must, animals under any degree of distress need to redirect efforts and energy to face the threats that are causing that situation. For productive cycles in an

industry, this means worse productive/growth ratios and incomes [81]. For research, this means introducing a new, undesirable variable in an experiment, which can and will disrupt the physiology of the animals, modify the results in a substantial way and adulterate any conclusions. It is for these reasons that animal welfare indicators are particularly important to maintain the repeatability principle of research, and especially for fish, animals whose ethology is very far away from that of humans, making the direct perception of distress in these animals a very complicated task.

The antioxidant status of rainbow trout livers followed very reasonable trends, even if they showed some behaviour differences among the studies. With the exception of Study 3, the levels of malondialdehyde (MDA) went down in many insect-based treatments, which is possibly the most enlightening and relevant result. MDA is a good indicator of membrane lipidic peroxidation and, consequently, of oxidative stress damage [81,82]. On the contrary, Study 3 followed an opposite tendency. As for enzymes related to the antioxidant response, they suffered different changes throughout the different studies and treatments, the case of activity increases being more frequent than the opposite. The activity of antioxidant enzymes can have different interpretations. First, an increase in the activity of enzymes such as superoxide dismutase (SOD) or glutathione peroxidase (GPx) could mean that the fish are theoretically better prepared againstoxidative stress. This is the most typical conclusion of insect-fed fish trials where the antioxidantresponse was analysed [83-85], and would be positive. However, that same increase in enzymatic activity could also mean that the fish are currently suffering from a prooxidative stress situation, which would generate a higher antioxidant response as well [82,86], and would be negative. On the other hand, a decrease in the activity of these enzymes could mean that the physiology of the fish is facing a lower level of oxidative stress [87,88], but it could also mean that it has been facing a chronic situation of oxidative stress, which would lead to a scenario of partial enzymatic depletion [86]. During the two first studies, the lower levels of MDA aim to the idea that the correct perspective should be a positive one, so increases should mean a stronger, more preventive status of the antioxidant response (for example, the case of SOD during Study 1, and for 5 % HI inclusion and 10 % TM inclusion), and decreases should mean that the fish are facing a lower level of oxidative stress (such as the case of GPx in Study 2). HI treatments did not follow the performance of TM treatments with the same intensity, which supports the idea that these levels of HI were closer to a theoretical limit of inclusion in the diets, as was reflected in the lower growth and ADC_{prot} levels of the highest HI inclusion (18 %) during Study 2.

Talking about the results of Study 3, the case would be different. TM diets supplemented with EO, and even the diet with partially defatted TM, showed a higher degree of lipidic accumulation in fillet (statistically significant for the low inclusion level of EO) and liver, and higher levels of long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs) in the fillets. LC n-3 PUFAs are the fatty acids (FAs) with the highest peroxidability indices, which means that they have the highest risk to produce lipidic peroxidation. In this way, the higher levels of MDA in diets enriched with EO are probably related to this and the lower levels of GPx could be related to a chronic depletion of glutathione [89,90] during the three months of the trial. However, the stable levels on the rest of the enzymes suggest that, indeed, the IM included in the diets might

have also played a role on the prevention of further oxidative damage. Another interesting detail to consider is that fish size/age can modify the antioxidant response in a significant way [91], so even though the reported results and the exposed arguments should make sense, it is possible that the differences among studies were altered by the different sizes as well.

The study of the innate immunological system offered fewer tangible conclusions than those of the antioxidant response, but they followed a similar trend. Some of the parameters that are related to a healthier status of the fish suffered an increase in their expression for insect-based treatments, such as the case of antiprotease and total Ig during Study 1. On the other hand, the expression of acid and alkaline phosphatases, which are known indicatives of tissue damage [92,93] went down during the first two studies. Again, the case of Study 3 was different. In general, there were no significant differences in the expression of most parameters during this last case. The study of skin mucus, which is the first biological barrier against pathogens, revealed higher levels of acid and alkaline phosphatases in the diet enriched with 3.09 % of EO, so all three cases followed the trend marked by the evaluation of the antioxidant status of the liver, showing a direct relation between phosphatases and MDA.

Before finishing this section, two more considerations should be noted. First, the levels of glucose and lactate of Study 2, and the levels of glucose of Study 3 remained unaffected. This means that even the most pronounced cases (lower growth with HI, and higher oxidative stress status of the diet enriched with 7.24 % of EO) were within healthy margins. It is reasonable to assume that these fish were not suffering from a real case of distress. Plasmatic levels of glucose and lactate are good indicators of animal welfare [94-96]. Even if these results are not meant to be absolute, they support other data like the overall good growth and physiological status of these fish. Lastly, IMs are supposed to be functional ingredients that can enhance the antioxidant and immunological status of the fish, but even though the evidence on this is wide, the real mechanisms on how this happens are yet to be discovered. For example, it has been described that different metabolites of chitin can enhance the immunological response of shrimp in different ways [97] and that, since chitin is a polymeric structure, different sizes of this molecule can trigger different effects [98]. It should be advised that until these mechanisms are properly identified and isolated, there will not be an effective way of using these ingredients properly as enhancers of these physiologic systems.

6. Effect on the quality of the final product

6.1. Fillet proximate and fatty acids composition

Inside the context of insect-fed fish, proximate composition of fillets and/or whole body is one among the parameters that offer the most controversial results. Talking solely about experiments with HI and TM for fish, the bibliography contains around 80 manuscripts that measured the proximate composition of either fish whole body or fillets. Among them, changes in fat seem to be the most common [69,99-105] while protein and/or ash show differences less often [63,99,102,105,106]. However, the three studies reported in this thesis followed one similar and consistent trend. Rainbow trout fillets developed a higher accumulation of fat for most

insect-based diets, but only the case of Study 3 showed true differences when comparing TM enriched with 3.09 % EO and non-enriched full fat TM, with control diet. This was probably because the fish reached a higher size and were fed for longer. The case of Study 1 did not show statistically significant differences, which could had happened because this study tested the lowest proportions of IMs in the diets, something which may have not allowed the expression of these parameters in a sufficient way. For Study 2, it is possible that the different size of fish interfered with the metabolism of the different nutrients, as can be seen in the unique differences observed for protein and ash. A feasible explanation for the changes in fat could be that insect-based diets had more diverse sources of fat. As it was mentioned in Study 3, fish count with different metabolic mechanisms that allow the development of metabolic "sparing effects" [107-110]. For this case, it is known that the metabolism of species like Murray cod (*Maccullochella peelii*) [111] and rainbow trout [112], prefer SFA and MUFA over LC n-3 PUFAs when it comes to using FAs to produce energy, provoking what has been referred to as "n-3 sparing effect". It has also been suggested that fish might be less efficient during the metabolism of alternative lipid sources [113,114]. As such, it would be reasonable to assume that this n-3 sparing effect could slow down the metabolism of fat when there are enough SFA and MUFA available, producing a partial surplus of fat and allowing its accumulation in tissues like muscle and liver in the long term. This would also explain why the partially defatted TM diet of Study 3 highlighted an intermediate level of fat, because the defatted IM used for this diet had also a small amount of insect fat within its composition.

Plenty of experiments have proven that a low concentration of LC n-3 FAs is transmitted through the fish diet [2,115-117], which was also reflected in Studies 1 and 3. One of the objectives of Study 3 was to put this concept to a test by trying to compensate the concentration of dietary LC n-3 PUFA. Partially defatted TM and high level (7.24 %) EO enrichment of TM were experimental diets formulated to have, functionally speaking, a similar composition to that of control diet. The diet with a low level (3.09 %) EO enrichment was supposed to stay within an intermediate level, while the non-enriched full fat TM diet was expected to act as a secondary control that would replicate the trend of Study 1. However, the presence of full fat IM in three of these diets, and even the presence of a partially defatted IM produced a metabolic imbalance, leading to the n-3 sparing effect that was previously mentioned [110-112]. In this way, the diets enriched with EO showed the highest values of LC n-3 PUFAs, and even the defatted TM diet got some values (eicosatetraenoic and docosapentaenoic acids) over the control diet. The treatment with non-enriched full fat TM, on the other hand, followed the expected trend that was shown in all full fat insect-based diets, with the highest values of oleic and linoleic acids, and the lowest values of LC n-3 PUFAs. It should also be noted that the EO enriched TM diets (both 3.09 and 7.24 %), presented a significant reduction of fish oil (52.8 and 87.8 %, respectively) in its formulation respect to control diet, which means that a purer status of LC n-3 PUFAs (eicosapentaenoic and docosahexaenoic acids) had a significant effect as well.

6.2. Instrumental and sensorial parameters of the fillet

Concerning the quality evaluation of fillets, a big part of the same parameters that were described and discussed in Study 3 were also analysed in the fillets of Studies 1 and 2, with two exceptions: 1) the instrumental texture analysis carried out in Study 3 was a complete texture

profile analysis (TPA), while for the experiments corresponding to Studies 1 and 2, due to the size of the fillets, only brittleness was analysed; 2) the sensorial analysis of cooked fillets was only carried out in Study 3 because in this case the fish reached a commercial size. The results of instrumental texture and colour corresponding to Studies 1 and 2 were not included in their respective articles due to publication requirements, so they are included in Appendix I of this thesis. The results of Study 3 are also included in Appendix I to facilitate the comprehension.

All results followed very similar and consistent trends among studies. Instrumental texture analyses showed no differences among treatments in any of the studies for any of the measured variables. Instrumental colour analyses showed no differences in Studies 1 and 2. A minor difference in lightness (L) was highlighted in Study 3 between the full fat insect-based diet the lower inclusion of EO, as well as a lower level of redness (a*) for the higher level of EO against the control. The direct sensorial analysis of raw fillets and whole fish did not highlight any differences among treatments for any of the studies. As for the direct sensorial analysis of cooked fillets, it was only described one statistically significant difference for brightness, being lower in fish fed with the highest level of EO enrichment (7.24 %) TM than in control and non-enriched full fat TM diet.

In general, all the results align correctly with what was described in Study 3. Even though there are few insect-fed fish trials in which these variables were measured in some way, the most frequent conclusion is that IMs do not alter the organoleptic profile of fish significantly [118-123]. When this happens, the changes are mostly related to the colour, either of the skin [124], the fillet [125-127], or both parameters [69,128]. However, there is also a small number of studies which described other significant changes, such as the flavour or the sensory profile [129,130]. Taking into account that the consumer opinion is a key pillar which rules over the last part of the production cycle, more research is encouraged to enlighten this point further.

During the elaboration of this thesis, a survey on the opinion of Spanish consumers about the use of insects as ingredients for fish has been initiated and is in progress to reach a significant number of respondents [Appendix II]. Interesting early findings can be derived on 70 respondents, from different age, sex and academic background groups, geographical origin (interior, coast or both) and relationship with aquaculture or fishing. The survey highlights different levels of rejection towards this kind of product for 44 % of the consumers. Inside that 44 %, 23 % of them would not try insect-fed fish. When asked a reason to support their opinion, 15-30 % could or would not give a real motive, 36 % consider that insects are unacceptable from a sanitary point of view, 23 % consider that insects should only be used as a last resource, and 46 % declare that they perceive insects as disgusting [131]. This enters in direct conflict with the previously mentioned about the results on the perception of the final product, possibly due to cultural prejudices in western countries such as Spain.

7. The current status of insect entrepreneurship, insect meals, and future perspectives of the sector

Like any other new and promising economic activity, insect entrepreneurship, or “entopreneurship” as it has been referred to in some publications, is currently in a situation of

high risks. A proof of this can be found in the high number of worldwide insect-related startups that tried to succeed but disappeared along the way [132]. However, due to the problems that it is trying to solve, this early-stage sector has a tremendous potential as well. Even though the production of insects is still in a pre-commercial phase widely dominated by research [133], it has also appealed the interest of investors [134,135] and promoted the development of some potent private companies [136,137]. The adoption of insect-related products, as well as the IM volumes required by animal feed companies, are still complicated entry barriers for small enterprises. It has been suggested that associating as cooperatives or specialising in precise intermediate processes for the manufacture of these products (insect breeding, larvae drying, IM defatting, production of alternative products like chitin or insect oil, among others) can be interesting methods to find a way into this novel industry [137]. This makes sense, because even if insects have interesting advantages from a productive point of view, this sector must compete with other economic activities and companies that have existed for decades or centuries. Although environmental sustainability is very important nowadays, it is far from being the only factor to consider. The current bibliography describes huge variations in the cost of different insect-related products, which can variate from less than 1 €/kg to 200 €/kg depending on the species, the processing and presentation of the final product and the target market [136,138]. However, for the biggest part, even the most "generous" among those costs could still be considered as quite expensive when compared to more standardised protein sources like soybean meal or fishmeal [139-141].

Talking about the future perspectives of the insect production industry, even though what was previously mentioned should give an idea that there is still some uncertainty, many reports tend to be optimistic about it in the mid to long term, but some appreciations could be made. A first approximation was given by the International Platform of Insects for Food and Feed (IPIFF), mentioning that 6,000 t of insect protein was produced in 2019 by European companies, with a projection of 0.5-2 million t for 2025, and between 1,5-5 million t for 2030 [142]. However, a recent report from RABOBank revealed that this mid-term objective was not yet achieved, stating that the production of insect protein was around 10,000 t in 2021 [135]. This should not be a big surprise, considering that between those two reports, the COVID-19 pandemic shook many economic sectors. Nevertheless, that same report, and other that are more recent, still give credit to the fact that the insect sector has a great future [135,137,143]. Actually, one of the most interesting target markets of the insect industry is pet feeds [134,135], another industry that, unlike other animal feed sectors, grew importantly even during the COVID-19 pandemic [144]. Because the pet feed industry works on a shorter scale than livestock feed industries, this allows the affordability of relatively higher costs, which could act as a bridge to favour the consolidation phase of the insect industry. This is indeed something that has already been considered, because as this sector matures, the cost of insect-related products should become more affordable. This should at the same time favour the adoption of IMs as realistic ingredients in animal feeds [134,135,137]. Other industries such as poultry and pig farming should help with this task as well. The current expectations aim to the idea that the adoption of IMs by aquaculture will be the most relevant factor for the consolidation of the insect farming industry, reaching predictive numbers of 500,000 t of insect protein in 2030 among the different animal feed sectors, and 200,000 t only for aquaculture [135].

Insects can, indeed, feed in different by-products and industrial/urban wastes that can hardly be given a productive use. Even though the current regulatory framework of the EU does not allow to introduce insects raised in this way into the human food chain, there are other uses for these products. Insect frass is known to be a great fertiliser, and other insect components such as chitin, protein and fat can be used for different ends that do not need to be related to the food chain, such as the production of biodiesel, bioplastics or cosmetics [136,137,145]. These are opportunities that could allow a private company to grow with lower costs, which would also enhance the development of the industry. Also, and even if this could be considered more of a long-term objective, there could be a possibility of producing insects fed on wastes that are not harmful for the human food chain, maybe through the development of a new technology, or simply if research discovered that some insects could process these by-products without creating contaminated products which mean risks for the food chain. Should that happen, this new industry would count with an invaluable advantage when compared to others.

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CHAPTER 7 – Conclusions

CONCLUSIONS

General conclusion: a partial replacement of fishmeal can maintain growth performance and feed efficiency of rainbow trout if the inclusion of insect meals remains within reasonable limits. From a pragmatic point of view, most of the physiological variables that were measured were not negatively affected. The quality of the final product is also unaffected by insect meals apart from diminishing the level of omega-3 in the fillets, a problem that can be solved through different strategies. Future research should deepen the biochemical mechanisms behind the effects on the immunological and antioxidant response, and the sparing effect of omega-3. This thesis highlights the potential of using insect meals as a sustainable alternative to traditional protein sources in feeds for rainbow trout.

Specific conclusions:

1) Rainbow trout growth was not disrupted in a significant way by the inclusion of insect meals when that inclusion stayed within reasonable levels. Black soldier fly (*Hermetia illucens*) showed a possible limit for this inclusion at around 10 % of the formulation, while an 18 % of yellow mealworm (*Tenebrio molitor)* still showed optimal levels of growth. [Studies 1, 2 and 3]

2) The digestibility of yellow mealworm protein was slightly superior to that of black soldier fly, and biometric indices remained mostly unaffected for both insect meals used, when compared with control diet. [Studies 1, 2 and 3]

3) Some results showed signs that insect meals suffer a more active digestive process in theintestine, but more research is needed on this topic. [Studies 1 and 2]

4) Fish fed with black soldier fly developed shorter intestinal villi, which could be a main cause of its lower growth. However, insect meals did not modify the intestinal histomorphology in most cases, even though they showed signs of reducing the pro- inflammatory effects of vegetable ingredients. [Studies 2 and 3]

5) Diets based on yellow mealworm could modify the intestinal microbiota in very differentways, and a promotion of lactic-acid bacteria was associated to insect fat. [Study 3]

6) Insect meals did not modify liver function (intermediary metabolism or histology) in a significant way. [Studies 1, 2 and 3]

7) Insect meals enhanced the immunological and the antioxidant response, but the reasons behind these effects remain mostly unknown. [Studies 1, 2 and 3]

8) The inclusion of insects in fish diets reduced the long chain omega-3 polyunsaturated fatty acid profile of the fish fillet, but it is possible to offer solutions to this through different strategies. [Studies 1 and 3]

9) The differences between fishmeal and insect meal fatty acids produced a metabolic imbalance, promoting an omega-3 sparing effect and a surplus of fat, allowing its accumulation in different tissues such as liver and muscle. [Study 3]

10) The perceived quality of the final product was mostly unaffected by insect meals, and most of these minor changes were related to the visual perception (colour and brightness). [Study 3]

11) Few differences were shown regarding the size of fish, the activity of amylase being oneof them (lower in insect meals treatments for mid stage fish, and higher for early stage) and the level of liver lipid accumulation being the other one (lower in all treatments for early stage fish). [Studies 1, 2 and 3]

12) Insect production is an industry with a great potential, but it is still at a very early stage, which is reflected in its relatively high costs and the prejudices shown by Spanish consumers. [Thesis discussion]

APPENDICES

APPENDIX I. Complementary data

Study 1

Table IA. Effect of dietary treatments on the texture and colour of rainbow trout fillets from Study 1 (instrumental analyses)

Instrumental analyses	**C**	**H1**	**H2**	**T1**	**T2**	**SEM**
Texture						
Brittleness (g)	107.82	128.53	128.72	109.94	135.37	16.57
Colour						
Luminosity (L*)	45.28	45.42	47.41	46.9	46.19	1.16
Redness (a*)	-2.1	-1.98	-2.14	-1.92	-2.31	0.12
Yellowness (b*)	6.04	7.14	6.29	5.54	5.82	0.69
Chroma (C^*_{ab})	6.4	7.41	6.67	5.9	6.31	0.69
Hue (H^o_{ab})	109.55	105.89	108.96	110.52	115.8	2.51

C: control diet (no fishmeal replacement); H1: 15 % fishmeal replacement with Hermetia illucens; H2: 30 % fishmeal replacement with Hermetia illucens; T1: 15 % fishmeal replacement with Tenebrio molitor; T2: 30 % fishmeal replacement with Tenebrio molitor. Values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet).

Table IB. Effect of dietary treatments on whole fish and fillets of raw rainbow trout from Study 1 (sensorial analysis)

Attributes in raw fish	**C**	**H1**	**H2**	**T1**	**T2**	**SEM**
Acceptability	2.28	2.91	2.52	2.68	2.43	0.5
Colour	3.10	3.42	3.99	3.8	3.94	0.58
Texture	1.52	1.57	2.23	1.91	1.36	0.53
Odour	1.96	2.7	2.96	2.52	2.93	0.5
Quality Index (QI)	2.89	2.22	2.83	3.22	2.94	0.55

C: control diet (no fishmeal replacement); H1: 15 % fishmeal replacement with Hermetia illucens; H2: 30 % fishmeal replacement with Hermetia illucens; T1: 15 % fishmeal replacement with Tenebrio molitor; T2: 30 % fishmeal replacement with Tenebrio molitor. Values are expressed as mean ± standard error of the mean (SEM; n=9 panellists per diet).

Study 2

Table IIA. Effect of dietary treatments on the texture and colour of rainbow trout fillets from Study 2 (instrumental analyses)

Instrumental analyses	**C**	**H18**	**T18**	**SEM**
Texture				
Brittleness (g)	128.36	127.49	103.31	18.04
Colour				
Luminosity (L*)	58.51	54.78	54.79	1.02
Redness (a*)	-2,49	-2,2	-2,3	0.11
Yellowness (b*)	7.73	6.14	7.34	1.05
Chroma (C^*_{ab})	8.23	6.56	7.73	0.96
Hue (H^o_{ab})	109.61	109.28	108.73	3.31

C: control diet (no fishmeal replacement); H18: 50 % fishmeal replacement with Hermetia illucens; T18: 50 % fishmeal replacement with Tenebrio molitor. Values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet).

Table IIB. Effect of dietary treatments on whole fish and fillets of raw rainbow trout from Study 2 (sensorial analysis)

Attributes in raw fish	**C**	**H18**	**T18**	**SEM**
Acceptability	3.08	2.78	3.2	0.53
Colour	4.77	3.65	4.78	0.42
Texture	2.28	2.53	2.48	0.57
Odour	2.51	2.73	2.25	0.54
Quality Index (QI)	4.96	5.9	3.79	0.75

C: control diet (no fishmeal replacement); H18: 50 % fishmeal replacement with Hermetia illucens; T18: 50 % fishmeal replacement with Tenebrio molitor. Values are expressed as mean ± standard error of the mean (SEM; n=12 panellists per diet).

Study 3

Table IIIA. Effect of dietary treatments on the texture of raw rainbow trout fillets from Study 3 (instrumental analysis)

Texture parameters in raw fillets	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
Brittleness	64.13	58.76	71.32	78.6	61.16	7.26
Hardness	182.34	144.48	156.93	179.01	121.7	23.49
Cohesiveness	0.44	0.49	0.42	0.4	0.45	0.03
Adhesiveness	-3.78	-3.88	-3.01	-2.82	-3.96	0.36
Elasticity	1	1	1.01	1.02	1.01	0.01
Chewiness	79.65	72.47	66.74	73.7	54.25	11.93
Gumminess	79.58	72.37	66.3	72.46	53.73	11.96

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. Values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet).

Table IIIB. Effect of dietary treatments on the colour of raw rainbow trout fillets from Study 3 (instrumental analysis)

Colour parameters in raw fillets	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
Lightness (L)	58.08^{ab}	57.16^{b}	59.05^{ab}	59.55^{a}	58.95^{ab}	0.54
Redness (a*)	-1.57^{a}	-1.74^{ab}	-1.80^{ab}	-1.74^{ab}	-2.08^{b}	0.09
Yellowness (b*)	7.64	6.48	5.84	6.9	7.05	0.52
Chroma (C^*_{ab})	7.83	6.74	6.13	7.13	7.38	0.48
Hue (H^o_{ab})	102.91	106.04	107.82	104.35	106.14	1.68

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. $^{a, b}$ *show statistically significant differences among diets ($p < 0.05$); values are expressed as mean ± standard error of the mean (SEM; n=4 tanks per diet).*

Table IIIC. Effect of dietary treatments on whole fish and fillets of raw rainbow trout from Study 3 (sensorial analysis)

Attributes in raw fish	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
Acceptability	2.87	2.7	2.78	1.97	2.4	0.19
Colour	5.37	5.09	5.5	5.99	5.48	0.27
Texture	2.74	1.98	2.16	1.87	1.85	0.2
Odour	2.75	2.68	2.55	2.67	2.7	0.43
Quality Index (QI)	3.12	3.77	4	3.85	4.69	0.42

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. Values are expressed as mean ± standard error of the mean (SEM; n=13 panellists per diet).

Table IIID. Effect of dietary treatments on cooked rainbow trout fillets from Study 3 (sensorial analysis)

Attributes in cooked fillets	**C**	**T**	**dT**	**TO1**	**TO2**	**SEM**
Appearance						
Brightness	9.04[a]	9.03[a]	8.68[ab]	8.93[ab]	7.27[b]	0.38
Exudate colour	7.48	7.53	8.06	7.88	8.17	0.66
Colour uniformity	7.84	7.86	6.88	7.06	6.31	0.67
Colour intensity	6.83	7.03	5.18	5.23	3.86	1
Odour intensity	6.58	5.95	5.05	4.7	5.88	0.53
Taste						
Sweet	2.78	1.56	1.17	2.58	1.29	0.61
Salty	2.12	2.08	2.11	1.81	2.65	0.35
Bitter	0.73	1.44	1.5	1.11	1.69	0.43
Acid	0.4	0.49	0.92	0.64	0.6	0.35
Texture						
Hardness	2.82	2.4	3.4	2.01	3.28	0.61
Juiciness	5.59	4.9	4.1	6.22	3.93	0.61
Fatty	2.24	3.28	2.75	2.6	2.15	0.48
Elastic/gumminess	4.14	3.22	4.8	3.57	3.77	0.57
Teeth adherence	3.82	2.94	4.56	3.82	4.23	0.55
Flavor						
Rancid	1.18	1.35	1.22	1.05	1.64	0.31
Vegetable	1.57	0.93	0.99	0.88	0.89	0.23
Mud/earthy	3.28	3.66	2.92	2.58	3.24	0.42
Sea	3.07	2.82	2.43	2.7	3.18	0.35
Fatty sensation	1.7	2.01	2.04	2.15	1.44	0.47

C: control diet (no fishmeal replacement); T: 50 % fishmeal replacement with Tenebrio molitor; dT: 50 % fishmeal replacement with partially defatted Tenebrio molitor; TO1: T diet supplemented with 3.09 % of omega-3 enriched algal oil (EO); TO2: T diet supplemented with 7.24 % of EO. [a, b] *show statistically significant differences among diets ($p < 0.05$); values are expressed as mean ± standard error of the mean (SEM; n=8 panellists per diet).*

APPENDIX II. Survey on the opinion of consumers

Introducción

El objetivo de la presente encuesta es obtener una aproximación al nivel de conocimiento de la población con respecto a varias temáticas relacionadas con el estado de la acuicultura actual. Algunas preguntas son relativas, por lo que no tienen una respuesta verdaderamente correcta o falsa; en base a esto, rogamos al encuestado que sea lo más sincero posible, y que no se sienta influenciado por el contenido de la propia encuesta.

Preguntas previas (Marcar con una X)

1) Edad (años)
☐ <18.
☐ 18-30.
☐ 31-50.
☐ 51-65.
☐ >65.

2) ¿En qué entorno se crió?
☐ Costa.
☐ Interior.
☐ Ambos.

3) ¿Personal o profesionalmente está relacionado con el sector de la acuicultura?
☐ Sí, en profundidad.
☐ Tengo alguna idea.
☐ Poco/me suena.
☐ No.
☐ No, pero sí soy cercano al sector de la pesca.

4) Género
☐ Hombre. ☐ Mujer.

5) ¿Cuáles son los estudios de más alto nivel oficial que Ud. ha cursado (con independencia de que los haya terminado o no)?
☐ Sin estudios.
☐ Estudios primarios (EGB, ESO, Certificado escolar).
☐ Estudios secundarios (BUP, Bachiller, Formación Profesional).
☐ Estudios superiores (Universitarios/Formación Profesional Superior).
☐ Máster/posgrado.
☐ Doctorado (o superior).

6) ¿Con qué frecuencia consume pescado?
☐ Diariamente.
☐ 2-3 veces a la semana.
☐ 1 vez a la semana.
☐ Nunca.
☐ NS (no sabe).
☐ NC (no contesta).

7) ¿Conoce usted el potencial de reciclaje para residuos orgánicos que posee una granja de insectos?
☐ Sí, en profundidad.
☐ Tengo alguna idea.
☐ Poco/me suena.
☐ No.

Cuerpo de la encuesta (Marcar con una X)

8) De todo el pescado consumido del mundo, ¿qué porcentaje cree que proviene de PISCIFACTORÍAS?
☐ <20 %.
☐ 21-40 %.
☐ 41-60 %.
☐ 61-80 %.
☐ >80 %.
☐ NS (no sabe).
☐ NC (no contesta).

9) Al ir a una pescadería, ¿se fija en si el pescado que quiere comprar procede de PESCA EXTRACTIVA o de PISCIFACTORÍA?
☐ Siempre.
☐ Habitualmente.
☐ A veces.
☐ Nunca.
☐ NS (no sabe).
☐ NC (no contesta).

10) En caso de hacerlo, ¿cuál elegiría ante un mismo precio?
☐ Pez de pesca extractiva. ☐ Pez de piscifactoría. ☐ NS (no sabe). ☐ NC (no contesta).

11) En las opciones siguientes ¿en qué grado ha influido cada una de ellas, en su decisión de la pregunta 10? (MARCAR UNA OPCIÓN EN TODAS)

	Poco/nada	Algo	Mucho	Exclusivamente
Es la opción más sana para el consumidor	☐	☐	☐	☐
Tiene menos antibióticos, hormonas y/o otros compuestos químicos/tóxicos/perjudiciales	☐	☐	☐	☐
Es más amigable con el medio ambiente	☐	☐	☐	☐
Los peces están más sanos y son más felices	☐	☐	☐	☐
El aspecto y sabor es mejor	☐	☐	☐	☐

Otro motivo (describir)

12) A lo largo de un ciclo productivo de una PISCIFACTORÍA, a los peces se les administra… (MARCAR UNA OPCIÓN EN TODAS)

	Ninguna cantidad (0 %)	Poca cantidad (1-30 %)	Cantidad intermedia (31-60 %)	Mucha cantidad (61-100 %)	NS (no sabe)	NC (no contesta)
Presa viva/fresca	☐	☐	☐	☐	☐	☐
Piensos	☐	☐	☐	☐	☐	☐
Tratamientos farmacológicos	☐	☐	☐	☐	☐	☐
Otro (describir)						

13) Si marcó alguna cantidad de "PIENSOS" en la pregunta anterior, de las siguientes opciones, ¿qué ingredientes piensa que se pueden encontrar en un pienso habitual para peces?

	Sí	No	NS (no sabe)	NC (no contesta)
Harinas vegetales	☐	☐	☐	☐
Harinas de pescado	☐	☐	☐	☐
Harinas de insecto	☐	☐	☐	☐
Otras harinas (carne, sangre…)	☐	☐	☐	☐

14) De las opciones que marcó 'SÍ', ¿en qué porcentaje cree que se encuentran en un pienso habitual para peces? (MARCAR UNA OPCIÓN EN TODAS)

	Ninguna cantidad (0 %)	Poca cantidad (1-30 %)	Cantidad intermedia (31-60 %)	Mucha cantidad (61-100 %)	NS (no sabe)	NC (no contesta)
Harinas vegetales	☐	☐	☐	☐	☐	☐
Harinas de pescado	☐	☐	☐	☐	☐	☐
Harinas de insecto	☐	☐	☐	☐	☐	☐
Otras harinas (carne, sangre…)	☐	☐	☐	☐	☐	☐

15) ¿Tomaría un pescado de piscifactoría que sabe que ha sido alimentado con…? (MARCAR UNA OPCIÓN EN TODAS)

	Sin dudar	Me lo pensaría	Probablemente no	De ninguna manera	NS	NC
Harinas vegetales	☐	☐	☐	☐	☐	☐
Harinas de pescado	☐	☐	☐	☐	☐	☐
Harinas de insecto	☐	☐	☐	☐	☐	☐
Tratamientos farmacológicos	☐	☐	☐	☐	☐	☐
Enumere algún otro producto o ingrediente que lo haría dudar en esta decisión						

16) Si en la pregunta anterior, e n la opción 'H ARINAS DE INSECTC ' contestó: 'M e lo pe nsaría', 'Probablemente no' o 'de ninguna nanera', ¿por q ué lo hizo? (MARCAR UI A OPCIÓN EI TODA)

	Totalmente en desacuerdo	Algo en desacuerdo	Ni en acuerdo ni en desacuerdo	Algo en acuerdo	Totalmente en acuerdo	NS	NC
Porque los insectos son sanitariamente inaceptables	☐	☐	☐	☐	☐	☐	☐
Porque transmiten enfermedades	☐	☐	☐	☐	☐	☐	☐
Porque los insectos son un último recurso, solo aprovechados en situaciones de necesidad	☐	☐	☐	☐	☐	☐	☐
Porque los insectos me dan asco.	☐	☐	☐	☐	☐	☐	☐
Otro motivo (describir)							

17) ¿Cuánta contaminación para el medio ambiente piensa que generan los siguientes tipos de granjas? (MARCAR UNA OPCIÓN EN TODAS)

	Ninguna	Poca	Algo	Bastante	Mucha	NS
De cerdos	☐	☐	☐	☐	☐	☐
De peces (piscifactoría)	☐	☐	☐	☐	☐	☐
De insectos	☐	☐	☐	☐	☐	☐
De vacas	☐	☐	☐	☐	☐	☐
De pollos (carne)	☐	☐	☐	☐	☐	☐
De huevos	☐	☐	☐	☐	☐	☐
De corderos	☐	☐	☐	☐	☐	☐