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Innovative protein sources in feed for salmonids: Effects on lipid metabolism, gut microbiota and fillet quality

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In memory of my father

La filosofia è scritta in questo grandissimo libro che continuamente
ci sta aperto innanzi a gli occhi (io dico l'universo),
ma non si può intendere se prima non s'impara a intender la lingua,
e conoscer i caratteri, ne' quali è scritto

Galileo Galilei, Il Saggiatore

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Summary

The purpose of aquaculture is to contribute to feed an increasing world population and to become more sustainable. Consequently, the aquafeed sector must adapt to supply more feed with less expensive and more eco-friendly ingredients. Insects have been studied as one of the promising and innovative protein sources. Evidence showed that their production leads to a circular bioeconomy, and generates positive societal externalities. Moreover, insects are considered fairly nutritious as aquafeed ingredients. Administering a new diet to fish rest on the assumption that animal welfare and final eating quality are preserved.

The present thesis analyses the effects of feeding the Salmonidae Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) with diets containing the larvae of *Hermetia illucens*, belonging to the Diptera order. The ultimate goal of the present research is to provide a meaningful insight into the decision making process for feeding and farming in order to optimise the overall production process. To fulfil this ambition, three topics were taken into consideration:

- gut microbiota, for its countless functions and widespread impacts that it can have on the hosts;
- lipid metabolism, for better understanding the laws behind the lipid constitution in the final product;
- final product quality, specifically addressing its fatty acid composition, for the importance of fish fatty acids in human nutrition.

The results on gut microbiota of either A. salmon or rainbow trout indicated that microbiota composition is modulated by dietary insect and that it differs depending on sample origin (mucosa or digesta). In addition to changes in composition, microbiota of fish fed diets containing insects was also marked by higher alpha- and beta-diversity. Additionally, mucosa samples tended to show lower alpha-biodiversity in comparison to digesta samples, fact that could be explained by positing that mucosa exercises an active selection.

The examinations on lipid metabolism were performed on rainbow trout by calculating the indices of fatty acid metabolism and the ratio of products:precursors in liver and fillet, as well as by qPCR gene expression of pyloric caeca, mid intestine and liver. The three approaches agreed on the fact that lipid metabolism was affected by the diets containing the insect. Specifically, pyloric caeca *elovl2* relative expression seemed to show an increased trend when rainbow trout were fed diets containing the insect. Also pyloric caeca and mid

intestine *fads2* expression seemed to increase following the increasing dietary insect.

Minor effects on protein, fatty acid composition and fillet quality were noticed. Fillet fatty acid profile of A. salmon and rainbow trout fed diets containing the insect partly resembled the dietary fatty acid profile, commonly by showing an increase in saturated fatty acids. The cardioprotective fatty acids, namely polyunsaturated of the n-3 series, were successfully retrieved in fish fillets in satisfying amounts, not reflecting the dietary content. In addition, consumers appreciated steamed A. salmon fillets irrespective of the dietary treatment, though small weaknesses related to color intensity and textural attributes were raised in the fillets of the insect group. All things considered, diets containing insect guaranteed a nutritious and appreciated food.

To conclude, as some bacteria may protect fish from pathogens as well as enhance physiological functions, it is highly desirable to delve into the effects that bacterial communities have on fish biology. Likewise, an interaction of microbiota with lipid metabolism cannot be excluded.

H. illucens larvae used as dietary source for A. salmon and rainbow trout modulated lipid metabolism, but the final eating quality of fillets was highly preserved. It seems probable that the modulation of other oily ingredients may play a role in generating such an outcome, and further studies would be necessary to unravel the underlying dynamics. Additionally, tailoring *H. illucens* larvae by rearing them on a substrate from marine origin could help improve the final result.

Riassunto

Il fine dell'acquacoltura è contribuire a sfamare una popolazione mondiale in continua crescita a, allo stesso tempo, migliorare la propria sostenibilità. Gli insetti sono considerati una tra le numerose fonti proteiche innovative, infatti la loro produzione ha la potenzialità di favorire lo sviluppo di una bioeconomia circolare che può produrre esternalità positive in campo sociale. Inoltre, gli insetti sono considerati correttamente bilanciati in nutrienti, tanto da essere annoverati tra gli ingredienti utilizzabili in acquacoltura. D'altro canto, la somministrazione di una nuova dieta ad un pesce presuppone che il benessere animale e la qualità finale del prodotto vengano preservati.

La presente tesi ha affrontato lo studio degli effetti che una dieta contenente larve del dittero *Hermetia illucens* può avere sui salmonidi salmone atlantico (*Salmo salar*) e trota iridea (*Oncorhynchus mykiss*). Lo scopo finale della presente ricerca è fornire gli strumenti per prendere le decisioni in allevamento e mangimistica, ottimizzando complessivamente il processo produttivo.

Sono state prese in considerazione tre tematiche:

- microbiota del tratto digerente, per le sue innumerevoli funzioni e per il diffuso impatto che ha sull'organismo ospite;
- metabolismo lipidico, per meglio comprendere le leggi dietro la costituzione lipidica che si ritrova nel prodotto finale;
- qualità del prodotto finale, con particolare attenzione alla sua composizione in acidi grassi, per l'importanza che gli acidi grassi caratterizzanti il pesce svolgono in nutrizione umana.

I risultati relativi al microbiota intestinale del salmone atlantico e della trota iridea hanno indicato che la composizione del microbiota è modulata dall'insetto presente nella dieta e, inoltre, che varia a seconda dell'origine del campione (mucosa o contenuto intestinale). Oltre ai cambiamenti della composizione, il microbiota dei pesci alimentati con insetti è stato caratterizzato da più elevate alfa- e beta-diversità. Infine, i campioni di mucosa tendevano a mostrare una alfa-diversità inferiore rispetto ai campioni di contenuto intestinale, fatto probabilmente spiegabile ipotizzando che la mucosa eserciti una pressione selettiva sulla comunità microbica.

Le indagini sul metabolismo lipidico sono state svolte su trota iridea calcolando gli indici del metabolismo degli acidi grassi presenti nei filetti e il rapporto prodotti:precursori in fegati e filetti, oltre ad analisi di espressione genica su cechi pilorici, intestino medio e fegato con qPCR. I tre approcci hanno concordato nell'affermare che il metabolismo lipidico era stato modulato dalle diete contenenti

l'insetto. In dettaglio, l'espressione relativa del gene *elovl2* nei cechi pilorici ha sembrato mostrare una tendenza in crescita parallelamente all'aumento del contenuto di insetto nelle diete. Anche l'espressione del gene *fads2* di cechi e intestino medio ha mostrato un debole trend parallelo all'inclusione di insetto nella dieta.

Effetti marginali sono stati rilevati sul contenuto proteico e degli acidi grassi dei filetti. Il profilo in acidi grassi dei filetti di salmone atlantico e di trota iridea alimentati con diete contenenti l'insetto è risultato solo parzialmente simile al profilo in acidi grassi delle diete; nella fattispecie, si è notato un aumento degli acidi grassi saturi nei filetti dei gruppi alimentati con l'insetto. Gli acidi grassi cardioprotettori, ossia i polinsaturi della sere n-3, sono stati ritrovati in quantità soddisfacenti nei filetti, vale a dire senza riflettere il contenuto nelle diete degli stessi. A supporto di ciò, i consumatori di un consumer test hanno apprezzato filetti di salmone atlantico cotti al vapore a prescindere dal tipo di dieta somministrata ai pesci, benché piccole debolezze legate all'intensità del colore e ad alcuni attributi della texture fossero state sollevate nei filetti appartenenti al gruppo alimentato con l'insetto. In conclusione, dalle analisi risulta che utilizzare diete contenenti l'insetto ha garantito l'ottenimento di un cibo apprezzato e nutriente.

Concludendo, dal momento che si ritiene che alcuni batteri proteggano il pesce da patogeni e che adiuvino le funzioni fisiologiche, sembra altamente consigliabile approfondire gli studi sugli effetti che le comunità batteriche hanno sulla biologia del pesce. Per questa ragione non si può escludere un'interazione del microbiota con il metabolismo lipidico.

Le larve di *H. illucens* utilizzate nelle diete per salmone atlantico e trota iridea hanno modulato il metabolismo lipidico, ma la qualità del prodotto finale è stata mantenuta elevata. È possibile che modulando gli ingredienti lipidici delle diete sia stato possibile ottenere questo ottimo risultato, studi più approfonditi potrebbero contribuire a spiegarne le dinamiche. Anche l'allevamento delle larve di *H. illucens* su substrati di origine marina potrebbe aiutare a migliorare il risultato finale.

List of abbreviations

ALA	α-linolenic acid
DHA	Docosahexaenoic acid
DI	Distal intestine
EPA	Eicosapentaenoic acid
FA	Fatty acid
FM	Fishmeal
H	*Hermetia illucens*
LC	Long chain
LNA	Linoleic acid
MI	Mid intestine
MUFA	Monounsaturated fatty acid
NGS	Next generation sequencing
OTU	Operational taxonomic unit
PC	Pyloric caeca
PI	Proximal intestine
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid

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Characterisation of the intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) fed with *Hermetia illucens* (black soldier fly) partially defatted larva meal as partial dietary protein source

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Leonardo Bruni, Gloriana Cardinaletti, Fabio Mina, Ike Olivotto, Giuliana Parisi, Basilio Randazzo, Giulia Secci, Francesca Tulli, Matteo Zarantoniello

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Introduction

Aquaculture production

Global hunger and a growing population on the one side, higher incomes and the awareness of the health benefits of eating fishery products on the other side, point the finger at the impelling urge to increase aquaculture production. Among the responses posited by the FAO, parallelly to relieve the pressure on natural resources, there is the suggestion to maximise "the ecosystem goods and services obtained from the use of oceans, inland waters and wetlands" (FAO, 2018).

This sentence capitalises the direction that aquaculture has undertaken in the last decades. Around a half of the fishery production is now composed of aquaculture products, that in 2016 peaked at 80.0 million tonnes of food "fish" (finfish, molluscs, crustaceans, aquatic animals; Figure 1), 30.1 million tonnes of aquatic plants and 37,900 tonnes of non-food products.

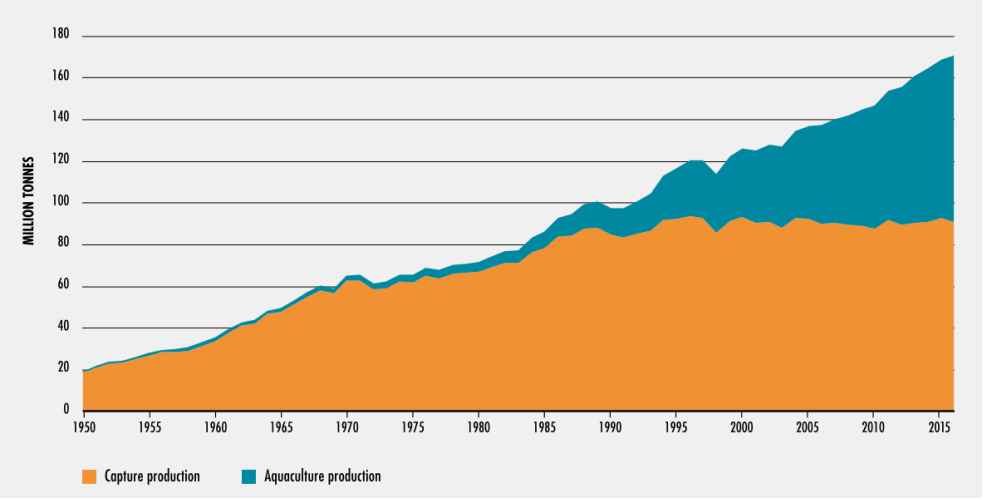

Figure 1. World capture fisheries and aquaculture production. Aquatic mammals, crocodiles, alligators, caimans, seaweeds and aquatic plants are excluded (FAO, 2018).

A number of 59.6 million people revolves around the aquaculture sector on a full-time, part-time or occasional basis. A sore point is gender inequality; in fact, women account for only 14% of all workers. On the other side, the proportion of capture fishery workers declined from 83% in 1990 to 68% in 2016 and the proportion of aquaculture workers complementary rose from 17 to 32%.

In 2016, A. salmon production summed up to more than 2.247 million tonnes (live weight), 4% of total finfish production, the ninth aquacultured species, the first seawater species and the first fish commodity by value. A. salmon is abundantly farmed in Norway, Chile, United Kingdom, Canada, Faroe Islands, Australia and other Countries. Norway alone produces 1,236,353 live weight tonnes, while Chile produces 614,180 live weight tonnes (FAO, 2019; Nelson et al., 2016). Global rainbow trout production went beyond 814,000 live weight tonnes and represented 2% of total finfish production (FAO, 2018), with Turkey, Chile, Norway, Peru, China and Italy being the first six producers and accounting for 60% of the global production (FAO, 2019). Even if the production volumes of A. salmon and rainbow trout are not comparable, rainbow trout is an important freshwater species in European Countries.

The production of A. salmon and rainbow trout has grown steadily and is projected to grow in the future (FAO, 2018). Also, global annual per capita fish consumption is expected to rise from 20.2 kg in 2015 (Figure 2) to 21.8 kg in 2025.

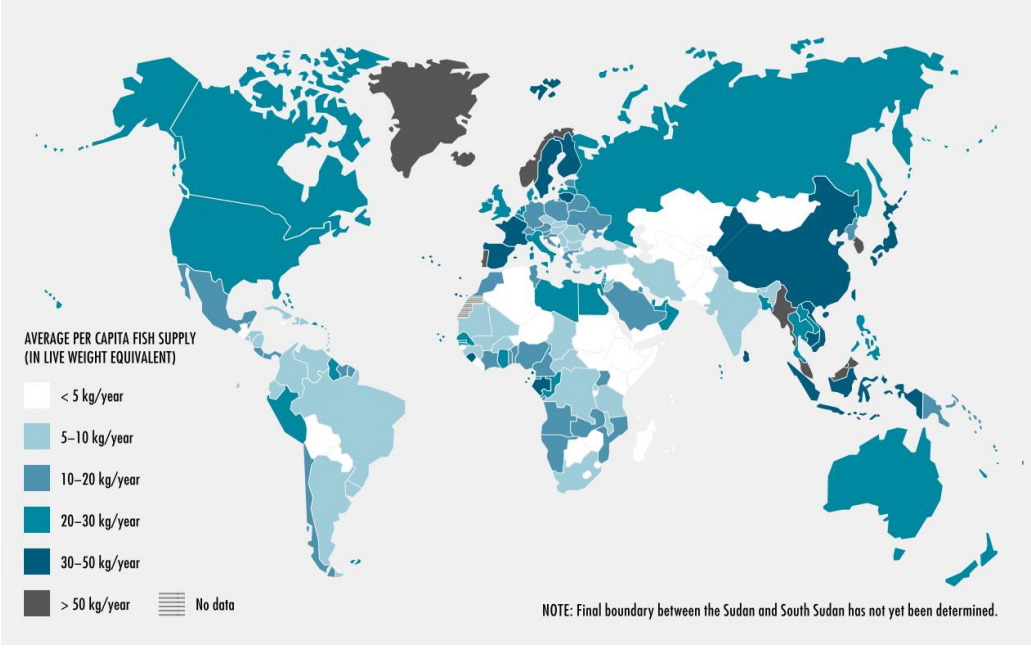

Figure 2. Apparent per capita fish consumption, average 2013-2015 (FAO, 2018).

The expansion of the aquaculture production, as a result, will require the expansion of the aquafeed industry. Considerations on this topic will be discussed in paragraph "Animal protein derived from farmed insects", while the next paragraph will concentrate on the biology of Salmonids.

Salmonids: anatomy, digestive physiology and lipid metabolism

Salmonidae family consists of a wide range of freshwater and anadromous Actinopterygii fishes, including 11 genera and more than 200 species. Native to cold waters rich of oxygen of the Northern Hemisphere, many species are now introduced worldwide for aquaculture and leisure purposes, as well as to restock wild populations. They typically have a fusiform body to more than 1.5 m length, present a typical adipose fin and own a tetraploid karyotype (Nelson et al., 2016), although triploid specimens can be found (Lien et al., 2016). Fishes popularly named salmons, trouts, charrs, whitefishes, ciscoes and graylings belong to this family (Nelson et al., 2016).

Salmo specimens specifically hail from the North Atlantic basin and include commercially important species, such as Atlantic salmon (*S. salar*), which is the first most produced seawater species and one of the most economically important aquacultured organism worldwide (FAO, 2018). On the other hand, *Oncorhynchus* spp. originate from the Pacific basin and comprise several economically important species, such as rainbow trout (*O. mykiss*), cherry or masu salmon (*O. masou*), amago salmon (*O. rhodurus*), pink salmon (*O. gorbuscha*), coho salmon (*O. kisutch*), chinook salmon (*O. tshawytscha*), chum salmon (*O. keta*) and sockeye salmon (*O. nerka*). Irrespective of the genus, those species that spend part of their adult life in saltwater are commonly termed "salmons", while those spending their lives in freshwater are usually termed "trouts". All Salmonids hatch in freshwater, usually from late summer to late winter in the Northern Hemisphere (Froese and Pauly, 2019; Nelson et al., 2016).

A. salmon and rainbow trout are carnivorous, and their feeding habit is reflected in their digestive system. The mouth is equipped with basihyal teeth and is followed by pharynx, an oesophagus with thick walls, a muscular J-shaped stomach, a linear intestine with thin walls, subdivided into proximal (PI), mid (MI) and distal intestine (DI) and ending with anus. After the pyloric sphincter, 50 to 100 pyloric caeca (PC) originate from the PI. The whole intestinal tract contributes to nutrient absorption, with a decreasing capacity along the length (Bakke et al., 2010; NRC, 2011). The proximal regions of the intestine largely contribute to the absorption of dietary lipids, especially in the form of free fatty acids (FA), lysophospholipids and monoacylglycerols, constituted by medium-chain or highly unsaturated FA (NRC, 2011; Turchini et al., 2009). Also, PC readily synthesise long-chain polyunsaturated FA (LC PUFA) (Bell et al., 2003). The MI conventionally begins after the last pyloric caecum. It primarily plays a role in digestion processes; here, saturated (SFA) and long FA are absorbed. An enlargement of the intestine marks the beginning of the DI, which is short and slightly more robust. This tract performs tasks in absorption of water, minerals, protein, lipids and carbohydrates, as well as roles in immunity and in osmoregulation (NRC, 2011).

Liver, pancreas, gallbladder and other smaller glands are organs annexed to the gastrointestinal tract and contribute to the digestive physiology by secreting digestive enzymes, bile, or by receiving and transforming nutrients from the

gastrointestinal tract. Nutrients are transported in the form of lipoproteins from the gastrointestinal tract to the liver, probably through the circulatory system as no lacteals are present (NRC, 2011). Among the numerous functions, the liver is the main actor in lipid metabolism (Tocher, 2003). For instance, the liver catabolises dietary lipids by β-oxidation, consequently producing acetyl-CoA, representing energy and the substrate for *de novo* lipid biosynthesis. Indeed, although PC, adipose tissue and muscle concur to the endogenous production of new lipids, liver plays the chief role.

SFA and monounsaturated FA (MUFA) are synthesised starting from acetyl-CoA and other carbon chains with the fatty acid synthetase and stearoyl CoA desaturase (also known as Δ9 desaturase) enzymes, respectively. Elongase enzymes then extend the carbon chain up to 20 atoms of carbon. However, as for all vertebrates, A. salmon and rainbow trout are unable to produce PUFA from SFA or MUFA. Benefiting from the essential linoleic (LNA, C18:2n-6) and α-linolenic (ALA, C18:3n-3) acids, Δ6 and Δ5 desaturase and elongase enzymes work in turn to produce LC PUFA. Although the same enzymes work both on the n-3 and on the n-6 FA series, they have higher affinity for the n-3 FA (Tocher, 2003). Docosahexaenoic acid (DHA, C22:6n-3) is produced via the so-called *Sprecher* pathway, that is, removing two carbons β-oxidising C24:6n-3 (Tocher, 2003). Recently, a desaturase showing Δ4 activity, enabling direct synthesis of DHA from eicosapentaenoic acid (EPA, C20:5n-3), was characterised in some Teleosts, but A. salmon and rainbow trout genomes seem devoid of this Δ4 desaturase gene; on the other hand, Salmonids are renowned to exhibit a relatively high Δ6 desaturase activity towards C24:5n-3 (Oboh et al., 2017), that could obviate to the lack of DHA production via Δ4 desaturation. As a matter of fact, Salmonids are not able to sustain the whole EPA and DHA need by endogenous production and, consequently, they show a dietary requirement.

Lipid homeostasis is governed by a conspicuous number of genes, in turn regulated by endogenous and exogenous conditions, for instance dietary lipids. Desaturases (*fads*$_s$), elongases (*elovl*$_s$), peroxisome proliferator-activated receptors (*ppar*$_s$) are some of those genes and are potentially expressed in all tissues. The *ppar*$_s$ are transcription factors that control the expression of genes playing roles in cellular FA uptake and desaturase activity (Tocher, 2003), in storage, mobilisation and burning of lipids, but also in glucose homeostasis, in respiration, in morphogenesis and inflammatory response (Janani and Ranjitha Kumari, 2015). Both quantity and quality of the dietary lipids affect *ppar*$_s$ expression. For example, fasting seemed to upregulate *pparα* and *pparβ* and downregulate *pparγ* expression in European sea bass (Rimoldi et al., 2016), a high SFA-diet increased liver *pparα* and *pparβ* expression in rainbow trout (Morash et al., 2009), SFA, MUFA, ALA, C20:4n-6 and DHA upregulated *pparα* expression of *in vitro* cultured rainbow trout hepatocytes, while LNA and EPA downregulated it (Coccia et al., 2014). Similarly, desaturase and elongase genes seem to be upregulated by a deficiency of LC PUFA or an increase in ALA (as reviewed by Tocher, 2015).

In summary, A. salmon and rainbow trout are productive and economically interesting species with a plastic lipid metabolism, whose physiology is relatively comprehensively known. To contribute to the expansion of the aquaculture production, an established knowledge is necessary to attain good outcomes in the highly mechanised, intensive and specialised aquaculture productive process.

Animal protein derived from farmed insects

As sustainability is gaining importance in aquaculture production, feed is among the important issues to be addressed. The traditional protein and lipid sources in intensive aquaculture have been marine-derived materials (fishmeal, FM, and fish oil), addressed to salmon and trout at a 7 and 2% share of consumption of total aquaculture feed by species group in the period 1995–2015, respectively (FAO, 2018). FM and fish oil are finite and, thus, not considered sustainable neither ecologically nor economically. The first substitutes used have been plant-derived sources, such as protein and/or oil of soybean, lupin, pea, wheat, corn, palm, rapeseed, followed by land-based animal protein, such as poultry by-products, bone and blood meals. Some of these categories bring about questions on their nutritive value, ease of supply, or on the environmental, economic and societal sustainabilities.

Additional alternative protein-sources are insects, marine macroalgae and plants, other marine organisms, single-cells grown on carbon sources and other organisms. The natural diet of Salmonids contains insects (van Huis et al., 2013) and these are currently used in aquaculture in Africa, China and other parts of Asia (EFSA Scientific Committee, 2015), as well as in Western Countries nowadays. Recently, the European Union (Regulation 2017/893/EC, 2017) admitted the use for aquaculture purposes of protein derived from seven farmed insect species: 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*).

Insects are considered promising dietary material for environmental, economic and societal reasons. Although studies on some of these issues are at their infancy, insects apparently need less feed, land and water than land-based animals, while at the same time giving the advantage to be farmed on organic waste streams, to produce protein and lipid sources qualitatively equivalent to other protein and lipid sources used in the aquafeed and to produce a waste that can be used as fertilizer, fostering a circular economy (van Huis et al., 2013). Indeed, when compared to other animals, they show having a higher proportion of edible material (e.g., 80% in crickets *vs* 55% in chicken and pigs) and a higher feed conversion efficiency (twice as efficient as chicken, four times as efficient as pigs and 12 times as efficient as cattle) (van Huis et al., 2013). There exist a few life cycle assessments in the literature; taking into consideration greenhouse gas emission, global warming potential, energy use and land use of *H. illucens*, *T.*

molitor or *Zophobas morio* farming. Researchers agree on the fact that insect production outperforms the production of lipid from rapeseed or protein from soybean and other land-based animals when it comes to land use and possibly water usage, greenhouse gas emissions and global warming potential, while energy consumption is the main critical element, due either to maintaining a warm rearing ambient or to the drying process needed to obtain meals from insect larvae, depending on the geographical location of the plant (Oonincx and de Boer, 2012; Salomone et al., 2017; Smetana et al., 2019). Only one study on economic outcomes of farming fish with diets containing insect meal is found in the literature; the paper focused on a specific case study on the marine species sea bass (*Dicentrarchus labrax*) fed with *T. molitor* and findings cannot be generalised (Arru et al., 2019). Lastly, a positive facet of insect farming is that insect harvesting and farming represent, in Developing Countries, an easily accessible source of both food and additional income, often offering a diversified livelihood thanks to the minimal technical knowledge and capital investment required (van Huis et al., 2013).

Among others, *H. illucens* (Diptera: Stratiomydae; Figure 3) is considered a promising species for its efficient bioconversion trait (van Huis et al., 2013) and for its fair and well described FA, amino acid and micronutrient contents (Barroso et al., 2014; Makkar et al., 2014; Sánchez-Muros et al., 2014). Lipids of *H. illucens* (H) are first of all composed of SFA and MUFA, a minimal part is made up of n-6 PUFA (Barroso et al., 2014; St-Hilaire et al., 2007a). For this reason, larvae of H meal are partially defatted to obtain an acceptable protein source to be put on the market. Technological development is needed to improve the defatting process, as the current commercial H meals contain up to 18% of total lipids (Kroeckel et al., 2012; Renna et al., 2017). On the other hand, the chemical composition of H, particularly its lipid component, is extremely variable depending on life stage, rearing substrate, handling and processing management. The flexibility of the lipid endogenous storage enables to tailor the fatty acid profile of H by rearing them on different substrates, in order to include desirable n-3 LC PUFA (Barroso et al., 2017; Liland et al., 2017; St-Hilaire et al., 2007a).

Figure 3. *Hermetia illucens* larvae (left) and adult (right).

Table 1 reviews the chemical composition of H found in original articles and reviews in the literature

Table 1. Chemical compositions of *Hermetia illucens* retrieved from the literature. Values are given as means ± standard deviation.

References	Notes	Crude protein	Ether extract	Fibres	Ash
Makkar et al., 2014	Larvae	42.1 ± 1.0	26.0 ± 6.0	7.0	20.6 ± 6.0
Barroso et al., 2014	Larvae	36.2 ± 0.3	18.0 ± 1.6	36.5 ± 1.0	9.3 ± 0.3
Barroso et al., 2014	Prepupae	40.7 ± 0.4	15.6 ± 0.1	24.0 ± 0.7	19.7 ± 0.1
Barroso et al., 2017	Larvae	53.8 ± 3.4	15.8 ± 0.2	18.7 ± 3.4	11.7 ± 0.1
Liland et al., 2017	Larvae fed on media containing 0% *Ascophyllum nodosum*	40.0 ± 0.9	33.8 ± 1.6 (total lipid)	*na*	5.1 ± 0.4
Liland et al., 2017	Larvae fed on media containing 100% *Ascophyllum nodosum*	41.3 ± 1.1	8.1 ± 0.9 (total lipid)	*na*	15.8 ± 0.7

Effect of diets on fish: gut microbiota

Administering diets containing novel ingredients brings about questions regarding fish welfare, farm economic outcome and final product safety, security and nutritive value. This paragraph will focus on an aspect of fish biology with possible implications on farm economic outcome as it touches fish welfare, safety and nutritive value: gut microbiota. The next paragraph will focus on the quality and nutritive value of the final product. In fact, as primary sector, one of the goals of aquaculture is to produce healthy and nutritious food to feed the world.

The microbiota of an animal is the set of all organisms living on its surfaces exposed to the external environment, including the gut environment. Here, bacteria account for the most numerous actors. Those found in the human intestine form a biomass of around 1.5 kg (Xu and Gordon, 2003) and their genomes contribute with around 540,000 genes (Evans et al., 2013), that is, more than 20 times the human genes, having a conservative estimate.

While researchers already call for understanding "how" the dialogue between host and guests is established, "who is there" is not entirely clear yet. In fact, studies on fish microbiota is a relatively new research field, rapidly expanding with the advent of new technologies. Generally, fish gut microbiota seems to be composed of Proteobacteria (62.5%), Firmicutes (15.2%), Bacteroidetes (6.0%), Actinobacteria (3.70%) and other less present operational taxonomic units (OTUs) (Sullam et al., 2012). Nonetheless, salinity, trophic level, habitat, host phylogeny are some of the several factors thought to shape the plastic gut microbiota (Sullam et al., 2012). Gut microbiota also varies depending on the intestinal tract, whether mucosa-associated vs digesta-associated bacteria are analysed (Gajardo et al., 2016) and on the diet administered to fish (Bakke-McKellep et al., 2007; Desai et al., 2012; Hartviksen et al., 2014; Ringø et al., 2015). In fact, the majority of the studies on fish microbiota focused on the role of diet on gut microbiota,

with the specific issue of administering alternative protein ingredients to carnivorous fishes (reviewed by Llewellyn et al., 2014).

The novelty of introducing insects in diets for fish did not go unnoticed when it comes to rainbow trout, while no studies on the microbiota of A. salmon fed with insects were found. Studies retrieved from the literature questioned the effect of dietary H meal, full-fat *T. molitor* or other insects on adult rainbow trout gut microbiota. The studies using H meal agree on the fact that the insect increases microbiota diversity and probably lactic acid bacteria (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019), while Antonopoulou et al. (2019) seems to suggest that *T. molitor* decreased gut microbiota richness. Intestine content of rainbow trout fed diets containing either *T. molitor*, *Gryllodes sigillatus*, *Blatta lateralis* or H meal was enquired for the presence of *Enterobacteriaceae*, *Clostridium leptum*, *C. coccoides*, *Lactobacillus sp.* and *Enterococcus sp.* by fluorescent *in situ* hybridisation (Józefiak et al., 2019); the authors found an increased number of these bacteria in the insect-fed groups. In spite of this partly shared feature, it is difficult to linearly compare the studies as the trials are marked by different experimental factors, such as insect origin, sample type collected, fish size, rearing duration, etc. Some of these issues are hereunder commented.

Regarding the diets, all the mentioned studies except for Huyben et al. (2019) formulated isonitrogenous, isolipidic and isoenergetic diets, with insect inclusion rates ranging up to 20% (Józefiak et al., 2019), 30% (Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019), 40% (Bruni et al., 2018) and 60% (Antonopoulou et al., 2019). The origin of the insects deserves a close look. The majority of the works examined the effect of commercial insects, whose substrate and rearing techniques are not fully disclosed. Moreover, larvae are usually defatted for fillet quality's sake, but Huyben and colleagues (2019) showed that feeding rainbow trout with different degrees of fat in the insect (hence in the diet) possibly modulated the gut microbiota; however, also dry matter, crude protein and fibre components differed between the dietary treatments and one cannot attribute the results to a single dietary ingredient. Similarly, the other mentioned studies, in order to maintain isonitrogenous, isolipidic and isoenergetic diets, also modified the inclusion levels of the ingredients to balance the protein and fat contribution of insect (Antonopoulou et al., 2019; Bruni et al., 2018; Józefiak et al., 2019; Rimoldi et al., 2019; Terova et al., 2019).

As for sample type, Bruni et al. (2018) collected mid and distal intestine together, but separated mucosa-associated from digesta-associated bacteria, finding that not only did the diet bestow different bacterial community, but also the mucosa and digesta communities seemed to differ, as previously found by Gajardo et al. (2016) in A. salmon fed with a commercial diet. Huyben et al. (2019) sampled distal intestine only and pooled mucosa and digesta together, probably

masking the difference between the two sample types. At an opposite position, Antonopoulou et al. (2019) decided to focus on mid intestine mucosa only. Józefiak et al. (2019) collected "intestinal contents" without specifying the tract of origin. Rimoldi et al. (2019) and Terova et al. (2019) stuck to Bruni et al. (2018)'s work making a pool of mid and distal intestine but separately analysing mucosa and digesta-associated microbiota; unfortunately, the results of the same rearing trial were published in two separate papers, one regarding mucosa, one regarding digesta, dooming the results on the different sample types not to be compared.

Finally, fish initial body weights were 53.4 g (Józefiak et al., 2019), 66.5 g (Rimoldi et al., 2019; Terova et al., 2019), 115.2 g (Antonopoulou et al., 2019), 178.2 g (Bruni et al., 2018) and 201.8 g (Huyben et al., 2019) and feedings lasted 35 days (Huyben et al., 2019), 71 days (Józefiak et al., 2019), 78 days (Bruni et al., 2018), 84 days (Rimoldi et al., 2019; Terova et al., 2019) and 90 days (Antonopoulou et al., 2019). Comparing the results generated from the different studies is not easy and may probably only lead to a description of some of the countless conditions encountered in real life.

To sum up, rainbow trout gut microbiota is in all likelihood susceptible to dietary inclusion of insect, however, this little review showed that profound knowledge of the specific factors directly affecting bacterial community is still lacking. Optimistically, once the changes of individual bacterial OTUs are revealed, functional studies can be implemented to unravel the mechanisms subtended to the host-guests dialogue. If correlation does not imply causation, one can assert that causation may be studied once correlation is successfully demonstrated (Peterson et al., 2009). Indeed, a thorough taxonomic description of the gut bacterial community composition usually leads to the identification of a core microbiota (Gajardo et al., 2016; Turnbaugh et al., 2009) and this is an essential step towards forthcoming functional, transcriptomic and metabolomic studies. The overarching goal is gaining mechanistic insights into microbiota's role in fish metabolism and into the principles leading its assembly and preservation, finally enabling to manipulate it to improve the health of the host (Collins et al., 2012; Merrifield et al., 2010; Roeselers et al., 2011).

The potential functions of gut microbiota include helping intestine digestive and absorption processes (Dehler et al., 2017; Ray et al., 2012), modulating lipid metabolism and energy balance (Falcinelli et al., 2015; Semova et al., 2012), but gut microbiota also establishes a dialogue with the central nervous system through the so-called microbiota-gut-brain axis (Lyte and Cryan, 2014) and impacts aging (Smith et al., 2017) and social behaviour (Archie and Tung, 2015). Last but not least, being at the interface between inner and outer environment, the mucosa-associated gastrointestinal microbiota constitute a line of defence against opportunistic bacteria (Verschuere et al., 2000). It is no surprise that microbiota was elected as virtual organ (O'Hara and Shanahan, 2006).

Methodology

Given its peculiar features, Llewellyn et al. (2014) predicted that research in fish microbiota would undergo an exponential growth of interest. As a matter of fact, the progress in microbiota studies was also boosted by the development and lower costs of the so-called next generation sequencing (NGS) techniques (Mengoni et al., 2015). NGS techniques enable metabarcoding surveys as well as whole-genome metagenomics, transcriptomics and other omics sciences. Briefly, though not exhaustingly, reviewing the analytical approaches of metabarcoding studies is necessary to give an overview of the countless checkpoints that researchers deal with from sampling to bioinformatic data processing. One could argue that each step of the analytical procedure introduces a bias. Blessedly, the difficulties encountered at each step allow this field to be dynamic and in relentless progression.

Sampling, DNA extraction, target gene choice and primer choice, PCR amplification, library preparation, sequencing, bioinformatics and biostatistics data processing: all these can be approached from different angles, with different aims and different tools (Kuczynski et al., 2012).

To start with, deciding which sample type to collect depends on the thought behind the project planning. As an example, deciding to collect only mucosa-associated bacteria precludes the possibility of further analyses on digesta-associated bacteria. Also, how one collects samples is crucial as it introduces the first contaminations from the sampling environment, and contamination is particularly odious when dealing with low microbial biomass; in such cases, the target DNA may be outcompeted by contaminating DNA in downstream steps. The sampling procedure seems to be standardised among the studies in the literature. In brief: wiping clean the fish abdomen with 70% ethanol, cutting the abdomen open, removing the whole intestine, separating the different tracts, opening them longitudinally, collecting the digesta, washing the mucosa twice or thrice in phosphate buffer saline and, finally, either storing the whole mucosa or the scraped layer. All steps must be performed close to a flame and tools need to be flamed during the work. The uncertainty remains of how close to sterile the procedure must be performed. Another hurdle in the first phases of microbiota analysis is the replication design, thoroughly commented in "Replicate or lie" by Prosser (2010).

Afterwards, DNA extraction easily introduces additional contamination and further bias. Contaminating DNA is present in every lab tools, reagents and extraction kits; introducing extraction negatives may help in subtracting the contaminating sequences (Salter et al., 2014). Moreover, different kits and different extraction protocols differently extract nucleic acids. Then, varying the temperature of some extraction step may deeply alter the results. Neglecting to comment on the particular difficulty of extracting DNA from spores, biodiversity of the most common microorganism *taxa* may be differently influenced by different extraction protocols (Kashinskaya et al., 2017). Also, the same extraction protocol may not be evenly efficient on all microorganisms. Last but

not least, the reduction of PCR inhibitors must also be addressed, for example by assessing PCR inhibition (Inglis et al., 2012).

When DNA is extracted and it comes to amplifying a target gene, the first action is the choice of the target. If bacteria are the target organisms, the target is usually the 16S rDNA gene for several reasons: it is universally distributed and infrequently horizontally transferred, it contains conserved regions (enabling the annealing of universal primers) and variable regions (enabling the observation of phylogenetic differences), its sequences and structure is described in several databases. One of the most important drawbacks is that it is not present in equal copies across species, undermining the possibility of assessing *taxa* abundance. Moreover, universal primers do not actually anneal identically to all known species; this flaw is partly bypassed using degenerate primers (Inglis et al., 2012). Lastly, the doubt remains that today's primers are built on the structure of known cultured microorganism species and the possibility that microorganisms with a slightly different DNA structure exist cannot be excluded *a priori*.

The target gene and primer set identified, amplification by PCR will introduce further bias in terms of mutations and chimeras. These can be limited by pooling multiple PCR runs and by reducing the number of PCR cycles. To reduce biases, new technological advancements also permit to run emulsion PCR, where, ideally, each DNA fragment is independently amplified (Inglis et al., 2012).

Library preparation, as each wet-lab step, is also prone to the introduction of contaminant sequences and requires the use of negative controls. In this phase, tag switching is also an issue and it can be controlled by tagging both ends of the amplicons, rinsing the PCR amplicons thoroughly during amplicon clean-up and reducing PCR steps during index and adaptor attachment.

Finally, NGS technologies and bioinformatic pipelines are various and each offers different costs and output, for instance read length, number of reads, run duration, error rate etc. (Mengoni et al., 2015). After the reads are obtained, a common bioinformatic pipeline would expect several steps: demultiplexing, quality control, consensus contigs production, demultiplexing, dereplication, OTU construction, chimera checking, taxonomic annotation, removal of non-target organisms and contaminant sequences, cleaning of tag-bleeding, OTU modifications, singleton or rare reads removal, and data transformation. QIIME2 is one of the several bioinformatics platforms condensing some of the numerous bioinformatic tools. Its modular architecture hopefully facilitates the reproducibility of microbiota analyses. Moreover, it offers visualization tools enabling investigating the results from different perspectives (Bolyen et al., 2019). The bioinformatic tools that do these actions proliferate and have many parameters that users need to adjust and adapt to their own datasets. Obviously, this flexibility is a manna and also a threat inducing to distorted results.

The research questions tested in metabarcoding surveys on the elaborated reads would then concern microbiota composition (who is there), richness and alpha and beta diversities (how many are there and how similar are my samples),

phylogeny (what evolutionary relationship do they have), correlation and differential abundance (which features, i.e., bacteria, are significantly more/less abundant in different experimental groups) (Gloor et al., 2017). Of particular notice is the concept that datasets produced by NGS techniques are compositional, a concept already well accepted in fields like geology, ecology and economics. In fact, the production of NGS datasets is subjected to the instrument capacity, that leads to obtaining relative abundances, or, in other words, to the fact that the sum of the relative abundances is always one (simplex constraint) and this undermines the assumption of independent data, thus precluding the use of standard statistical tests.

To conclude, by virtue of the several control knobs in microbiota studies, it seems essential to carefully and deeply describe and discuss the methodological part of the experiment, not least by publishing the script of the bioinformatics pipeline. Only by doing so the hypothesis can be verified and shared; in other words, reproducibility would be fulfilled.

Effect of diets on fish: nutritional quality

As outlined before, final product quality must be thoroughly investigated when diets containing novel ingredients are administered. Fish quality is meant as its set of physical, chemical and sensorial attributes, and its evaluation is a *sine qua non* both for business outcome and for human nutrition and health. Aquaculture products are well-known for their high n-3 LC PUFA content. These FAs are accumulated in the fillets, drawn from the diet and, in Salmonids and freshwater fishes, from a relatively small endogenous production (Tocher, 2003). Among the LC PUFA, EPA and DHA are the most valued FAs, known to diminish cardiovascular risk and inflammatory disorders and possibly depression in humans (Rosenlund et al., 2010). These specific features also secure a good market segment to aquaculture products.

The first studies on the effect of dietary insects on fish date back to the 20th Century (Bondari and Sheppard, 1981), but research started blossoming more recently (Madu and Ufodike, 2003; St-Hilaire et al., 2007b). When feeding rainbow trout with diets containing H meal, growth performance and digestibility are not a source of problems as far as the inclusion level is below 40% and the diet formulation is carefully designed (Renna et al., 2017), while digestibility in freshwater A. salmon slightly diminishes when H meal is included at a 60% level, although growth and feed conversion ratio are not negatively affected (Belghit et al., 2018). Recently, Iaconisi et al. (2019) found that total amino acid and free amino acid composition of rainbow trout was altered by administering diets containing *T. molitor*.

But the major problem when feeding insects to fishes pertains to their lipid component. By including insect meal in aquafeed, SFA and MUFA are also administered to fish, and, consequently, the animals accumulate those FAs in the edible portion, resulting in a change of the qualitative characteristics in the

direction of mirroring the FA profile of the diet (Belghit et al., 2018; Iaconisi et al., 2018; Mancini et al., 2017; Renna et al., 2017). To ensure fillets with a nutritious FA profile, two approaches have been studied. Firstly, given the high plasticity of the insect lipid metabolism, larvae were reared on substrates rich in n-3 LC PUFA, like *Ascophyllum nodosum* (Liland et al., 2017) or fish offal (St-Hilaire et al., 2007a), with the result of accumulating those FAs to a certain extent. The insects reared on these substrates were then reduced to protein meal and administered to A. salmon (Belghit et al., 2019) or rainbow trout (Sealey et al., 2011). The second approach considered a thorough modulation of the lipidic ingredients of fish feed, specifically augmenting fish oil inclusion level; this approach was implemented in diets for A. salmon (Belghit et al., 2019), but the spontaneous question arises if the dietary fish oil addition compromises the sustainability accomplished by replacing fishmeal with insect meal.

Finally, studies evaluated the effect of administering insects on the quality of cooked fish. FA profile and nutritional indices of cooked rainbow trout fillet strictly mirrored the values of the raw fillets; as a consequence, the consumers should increase the intake of fillets of fish fed with dietary insect to meet the suggested intake of EPA and DHA (Iaconisi et al., 2018; Secci et al., 2019). On the other hand, studies agreed by stating that minor changes in the fillet sensory quality are established in cooked A. salmon or rainbow trout fed H meal in comparison to fish fed commercial-like diets, however, no positive nor negative judgement was given to the changes found (Belghit et al., 2019; Borgogno et al., 2017).

As exposed, studies generally agree on the fact that dietary administration of H meal can replace fishmeal to some extent, while it poses questions regarding the final product eating quality, specifically regarding the valuable EPA and DHA content. As A. salmon and rainbow trout are among the few species able to synthesise endogenous EPA and DHA, it is of utmost importance to obtain a complete knowledge over the use and effects of insects as food and feed and over the biological changes that insect administration provokes in fish.

Focus: EPA and DHA shortage

Although human beings possess the set of genes encoding desaturase and elongase enzymes, they cannot reach their physiological demand by endogenous biosynthesis; consequently, EPA and DHA are listed among the essential FA. As a matter of fact, the marine environment is the source supplying the majority of these FAs, mainly from microalgae as the first producers in the trophic chain. For humans, there is no dietary reference intake or adequate intake for EPA and DHA, there rather exist recommendations ranging from 250 to 1,000 mg EPA+DHA per day (as reviewed by Tocher et al., 2019), with the most accredited references suggesting to ingest 500 mg EPA+DHA per day to prevent cardiovascular diseases (Cunnane et al., 2004). It follows that, amounting the world population to around 7 billion people, the yearly global demand for n-3 LC PUFA sums to 1.27 million tonnes (Tocher, 2015).

Two problems are in plain sight. On the human health side, the average estimated intake per person is 163 mg/day (Micha et al., 2014), well below the recommended intake thresholds. On the primary sector side, the calculations of global n-3 LC PUFA deficit span from 0.4 to more than 1 million tonnes (as reviewed by Tocher et al. (2019). The supply of EPA and DHA mainly comes from the aquaculture production at present. While an urgent call was raised for developing molecular technologies for producing *de novo* EPA and DHA, the need for thorough harvesting and recycling was also restated, although this "19/20th century approach" is not considered the solution to the problem (Tocher et al., 2019).

Nonetheless, the fish industry produces several by-products that are partly readdressed to the fish industry as fishmeal and fish oil, partly wasted. A source of n-3 LC PUFA that must not be neglected is the skin, whose composition and use is disregarded in the literature. Typing "fish skin" in literature search engines, the results show studies on biology, immunological functions, antioxidant properties and gelatine extraction and properties. Very few studies dedicated to the lipid content of the fish skin.

Njinkoué et al. (2002) revealed that the skin of *Cephalopholis taeniops*, *Sardinella aurita* and *Sardinella maderensis* during the upwelling period contained 2.4, 24 and 26% lipids (fresh weight), and EPA and DHA summed together accounted for about 10, 12.9 and 24.7% of the total FAs, respectively. To my knowledge, that is the sole study questioning skin for nutritional purposes and proposing its use as cooking oil. de Castro et al. (2007) analysed carp (*Cyprinus carpio*), Nile tilapia (*Oreochromis niloticus*) and tambacu (tambaqui, *Colossoma macropomum*, × pacu, *Piaractus mesopotamicus*) and found that total lipid content in fish without skin was 63, 39 and 71% lower than fish with skin, respectively. Again, Indian mackerel (*Rastrelliger kanagurta*) seemed to accumulate about 74% PUFA (on total FA) in skin, and this tissue also accumulated more EPA and DHA than flesh, heads or viscera (Sahena et al., 2010). Only two studies on rainbow trout could be found. Fish fed diets containing fish oil or 50 and 100% fish oil replaced by camelina oil, from 45 g to 173 g, showed a skin total lipid content (on wet weight) ranging from 2.2 to 3.7%, and EPA and DHA ranging from 9.1 to 2.0 and from 9.5 to 5.4 (mole percentage of total FA), respectively. The results indicate that skin contained a fair content of those FAs, even if dietary oil had an impact on skin FA profile (Hixson et al., 2014).

The findings exposed above suggest delving into the possible exploitation of skin for alimentary purposes.

Aims of the study

The increasing world population, the higher incomes, the awareness of the beneficial features of fishery products lead to a considerable growth of the demand and production of the aquaculture sector and of the serving aquafeed sector. In the perspective of a sustainable development, to supply the sector with abundant and qualitatively good feed material, the conventional dietary ingredients have been gradually replaced by less expensive and more eco-friendly ingredients. Insects embody a promising answer for being highly nutritious, for showing traits leading to a circular bioeconomy and for their societal externalities. However, when administering a new diet to fish, concerns regarding animal welfare and end-product quality arise.

In fact, changing the feed of an animal can potentially impact largely on its physiology and on its final quality, therefore preventing detrimental changes in both fields is crucial. For this reason, the present thesis addressed the effects of feeding the Salmonidae Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) with diets containing *Hermetia illucens* larvae. Close attention was paid to:

- gut microbiota, studied to show a picture of the eventual modifications due to dietary changes and to enable future well-aimed studies with advanced omics techniques to understand the functions of the dwelling microorganisms;
- monitoring end-product quality and its constitution, with specific concern about fatty acids, the feather in the cap of fishery production, an element that the consumers mainly seek for when they buy fish.

The final aim of the present research is to produce information on the changes in the gut microbiota and in the final quality of Salmonidae, as to give tools to assist in decision making for feeding and farming, overall optimising the production process.

Materials and methods

Ethical statement

To fulfil the project aims, the trials were designed in compliance with the ethical standards approved by the current European Directive 2010/63/EU on the protection of animals used for scientific purposes. Diet formulation, fish growth and sampling were performed at partner Research Institutes with certified scientists.

Overview of the studies

Table 2 shows the studies performed during this thesis. Specifically, trials 1 and 2 were designed with the aim of learning and fine-tuning analytical methods to be used in the other larger studies.

Table 2. List of the trials, corresponding papers and categories of the analyses performed.

Trial	Species	Dietary ingredient of the treatment group	Gene expression	Gut microbiota	Quality	Paper
1	*Oncorhynchus mykiss*	defatted *Hermetia illucens* larvae		x		1
2	*Salmo salar*	defatted *Hermetia illucens* larvae		x		2
2	*Salmo salar*	defatted *Hermetia illucens* larvae			x	3
2	*Salmo salar*	defatted *Hermetia illucens* larvae			x	4
3	*Oncorhynchus mykiss*	macroalgae			x	5
4	*Danio rerio*	full-fat *Hermetia illucens* larvae	x		(x)	6
5	*Oncorhynchus mykiss*	full-fat *Hermetia illucens* larvae	x		x	7
5	*Oncorhynchus mykiss*	full-fat *Hermetia illucens* larvae	x		x	8

Paper 1 considered the gut microbiota of rainbow trout fed defatted H larvae meal. The candidate contributed to conceiving the idea and to designing the experiments, performed most of the experiments and statistical computations, and took the lead in writing the manuscript. Published article: *Characterisation of the intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) fed with* Hermetia illucens *(black soldier fly) partially defatted larva meal as partial dietary protein source*, Aquaculture, https://doi.org/10.1016/j.aquaculture.2018.01.006.

Paper 2 considered the gut microbiota of A. salmon fed with diets containing defatted H larvae meal reared on a media containing *A. nodosum* and organic

wastes, the same diets used in studies 3 and 4. The paper has two equally contributing first authors. Specifically, the candidate suggested the idea to the co-authors, contributed to preparing the samples and to carrying the experiments, took lead in writing introduction and results; results were equally discussed by the two first authors. The manuscript is in the last phase of writing and will soon be forwarded to the co-authors.

Paper 3 considered the qualitative traits of raw Atlantic salmon (*Salmo salar*) fed with diets containing defatted H larvae meal reared on a media containing *Ascophyllum nodosum* and organic wastes, the same diets used in studies 2 and 4. The candidate contributed to preparing the samples, carried out the statistical computations, discussed the majority of the results and took the lead in writing the manuscript. The article is in press: *Total replacement of dietary fish meal with black soldier fly (*Hermetia illucens*) larvae does not impair physical, chemical or volatile composition of farmed Atlantic salmon (*Salmo salar *L.)*, J Sc Food Agric, https://doi.org/10.1002/jsfa.10108.

Paper 4 considered the qualitative traits of cooked A. salmon fed with diets containing defatted H larvae meal reared on a media containing *A. nodosum* and organic wastes, the same diets used in studies 2 and 3. The candidate contributed to preparing the samples and discussing results, and revised the final version of the manuscript. The manuscript is in revision status among the co-authors.

Paper 5 considered the administration of commercial diets to rainbow trout (*Oncorhynchus mykiss*) and investigated the final product quality. The PhD candidate carried out most of the experiments, produced and discussed the majority of the results, and took the lead in writing the manuscript. The manuscript is in revision status among the co-authors

Paper 6 considered the rearing of zebrafish (*Danio rerio*) fed with diets containing full-fat *Hermetia illucens* (H) larvae meal, the same diets later fed to rainbow trout in studies 7 and 8; zebrafish was used as it is a model organism with fast reproduction rate and growth and vast knowledge on its biology is available. The analytical methods were verified and then used to design the other trials. The candidate helped in sample preparation and in carrying out fish rearing, contributed to gene expression result production and discussion, then helped in the introduction and discussion sections of the manuscript. Published article: *Partial dietary inclusion of* Hermetia illucens *(Black Soldier Fly) full-fat prepupae in zebrafish feed: Biometric, histological, biochemical, and molecular implications*, Zebrafish, https://doi.org/10.1089/zeb.2018.1596.

Paper 7 considered the growth and gastrointestinal integrity of rainbow trout with diets containing full-fat H larvae meal, the same diets used in studies 6 and 8. The candidate contributed to laboratory analyses. Published article: *Effects of graded dietary inclusion level of full-fat* Hermetia illucens *prepupae meal in practical diets for rainbow trout (*Oncorhynchus mykiss*)*, Animals, https://doi.org/10.3390/ani9050251.

Paper 8 considered qualitative traits, liver FA composition and the expression of genes related to lipid metabolism in liver, pyloric caeca and mid intestine of rainbow trout fed with diets containing full-fat H larvae meal, the same diets used in studies 6 and 7. The candidate conceived the idea of investigating lipid metabolism in parallel to fish quality, planned the gene expression experiments and performed the statistical computations of the data. Manuscript writing was led by him. The manuscript is in revision status among the co-authors.

Diets

All diets were formulated to be isonitrogenous, isolipidic and isoenergetic and responding to the specific fish species needs. Tables 3 to 6 show the diets used in the experiments, also described in detail in the annexed Papers 1-8.

For ***Paper 1***, three diets were formulated: a control diet with fishmeal as main protein source (Hi0), and diets Hi25 and Hi50 where 25% and 50% of fishmeal, respectively, was replaced with partially defatted meal of H larvae.

Table 3. Formulation (% fresh matter) and proximate composition (g/100 g dry matter) of the experimental diets fed to rainbow trout in trial 1 (from Renna et al., 2017).

	Hi0	Hi25	Hi50
Ingredients (%)			
Fishmeal[1]	60	45	30
Hi meal[2]	0	20	40
Wheat meal	4	4	4
Wheat bran	9	6	3
Starch (D500)	15	15	15
Fish oil	9	7	5
Mineral mixture[3]	1.5	1.5	1.5
Vitamin mixture[4]	1.5	1.5	1.5
Chemical composition			
Dry matter (DM, g/100 g)	96.07	94.93	95.63
Crude protein (g/100 g DM)	45.20	44.86	45.00
Ether extract (g/100 g DM)	15.86	15.74	15.81
Ash (g/100 g DM)	11.40	11.43	10.11
Chitin (g/100 g DM)	0	1.05	2.09
Gross energy (MJ/kg of DM)	21.71	22.35	22.60

[1] Fish meal was purchased from Corpesca S.A. (Santiago, Chile). Proximate composition (% as-fed basis): 90.4 dry matter; 66.7 crude protein; 8.3 ether extract; 14.9 ash.

[2] *Hermetia illucens* larvae meal purchased from Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, Germany).

[3] Mineral mixture (g or mg/kg diet): bicalcium phosphate 500 g, calcium carbonate 215 g, sodium salt 40 g, potassium chloride 90 g, magnesium chloride 124 g, magnesium carbonate 124 g, iron sulfate 20 g, zinc sulfate 4 g, copper sulfate 3 g, potassium iodide 4 mg, cobalt sulfate 20 mg, manganese sulfate 3 g, sodium fluoride 1 g (Granda Zootecnici, Cuneo, Italy).

[4] Vitamin mixture (IU or mg/kg diet): DL-αtocopherolacetate, 60 IU; sodium menadione bisulfate, 5 mg; retinylacetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; Vitamin B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg (Granda Zootecnici, Cuneo, Italy).

For ***Papers 2***, ***3*** and ***4***, four diets were formulated: a control diet (IM_0, 0% of fishmeal substitution) and three diets with increasing substitution levels of the fishmeal with H larvae meal (IM_{33}, IM_{66} and IM_{100}; 33, 66 or 100% substitution levels, respectively). The H larvae were grown on media partially containing *Ascophyllum nodosum* mixed with organic waste streams (60:40).

Table 4. Formulation (% fresh matter) and proximate composition (g/100 g dry matter) of the four experimental diets (IM_0, IM_{33}, IM_{66} and IM_{100}) fed to Atlantic salmon (*Salmo salar* L.) in trial 2 (from Belghit et al., 2019).

	IM_0	**IM_{33}**	**IM_{66}**	**IM_{100}**
Ingredients (%)				
Fishmeal LT94	10	6.67	3.33	0.0
Insect meal	0.0	4.91	9.84	14.75
Soy protein concentrate	25	25	25	25
Corn gluten meal	7.5	7.5	7.5	7.5
Wheat gluten meal	3.35	4.51	5.7	6.88
Pea protein concentrate 55	8.8	6.8	4.8	2.84
Fish oil	10.18	11.70	13.23	14.76
Rapeseed oil	20.95	18.86	16.79	14.73
Binder	12.32	12.08	11.72	11.24
Additives	1.89	1.96	2.1	2.29
Yttrium	1.0	1.0	1.0	1.0
Proximate composition (%)				
Dry matter	93	93	94	95
Crude protein	38	38	39	39
Crude lipids	29	29	29	29
Ash	4.6	4.6	4.5	4.5
Carbohydrates	11.6	11.5	11.5	11.4
Gross energy (MJ/kg)	24.6	24.9	24.8	25.0
TBARS (nmol/g)	3.0	3.4	4.2	4.9

For ***Paper 5***, three diets were formulated: a fishmeal-based positive control diet (C+), a vegetable protein-based negative control diet (C-) and a diet similar to C- where 5% of soybean concentrate was replaced by a commercial blend of seaweeds.

Table 5. Formulation (% fresh matter), proximate composition (% on as is basis) and fatty acid class content (g/100 g total FAME) of the extruded experimental diets fed to rainbow trout in trial 5.

	C+	C-	T
Ingredients (% as fed)			
Fishmeal 999	53.2	6.2	6.2
CPSP 90 IDR	6.0	6.2	6.2
Wheat gluten	8.5	10.3	10.3
Corn gluten	0.0	12.4	12.4
Soy protein concentrate	8.5	10.3	10.3
Commercial blend of seaweeds	0.0	0.0	5.2
Soy meal extract (48%)	0.0	25.8	20.6
Fish oil	15.1	8.2	8.2
Rapeseed oil	6.0	11.9	11.9
Idropalm	2.4	3.6	3.6
Vitamin premix	0.1	0.1	0.1
Oligomineral premix	0.1	0.1	0.1
Dicalcium phosphate	0.0	2.6	2.6
DL-Methionine	0.0	0.5	0.5
Lysine-HCL	0.0	1.5	1.5
Betaine	0.0	0.3	0.3
Total	100.0	100.0	100.0
Fish protein (% of crude protein)	70	10	10
Fat coming from fish (% of crude fat)	70	35	35
Proximate composition (% on as is basis)			
Crude protein	41.2	41.1	41.0
Crude fat	24.4	24.2	24.3
Fatty acid classes (g/100 g total FAME)			
SFA	22.856	27.433	29.945
MUFA	42.892	40.190	40.666
PUFAn-6	14.959	19.920	18.119
PUFAn-3	18.472	11.936	10.774
n-3/n-6	1.24	0.60	0.60

For ***Papers 6***, ***7*** and ***8***, three diets were formulated: a control diet based on fishmeal and purified protein-rich vegetable ingredients with 0% H inclusion (H0) and two experimental diets with graded substitution levels of H meal at 25% (H25) and 50% (H50) in place of fishmeal.

Table 6. Ingredients (g/kg fresh matter), proximate composition (g/100 g), total lipids (g/100 g) and fatty acid profile (% of total FAMEs) of the experimental diets fed to zebrafish in Paper 6 and to rainbow trout in Papers 7 and 8.

	H0	**H25**	**H50**
Ingredients (g/kg)			
Chile prime fish meal[1]	420	315	210
Protein-rich vegetable ingredients[2]	110	156	200
H meal[3]	-	105	210
Wheat flour[4]	290	268	255
Fish oil	70	40	28
Palm oil	70	75	56
Mineral[§] and Vitamin[#] supplements	20	20	20
Binder	20	20	20
L-Methionine	-	1	1
Proximate composition[*] (g/100 g)			
Moisture	4.24 ± 0.03	5.49 ± 0.03	5.31 ± 0.18
Crude protein	40.27 ± 0.45	39.98 ± 0.37	40.16 ± 0.39
Ether extract	18.63 ± 0.27	18.56 ± 0.14	17.68 ± 0.20
Ash	14.30 ± 0.28	14.20 ± 0.23	14.13 ± 0.31
Gross energy (MJ/kg)	22.10 ± 0.11	22.30 ± 0.03	21.28 ± 0.06
Total lipid g/100g	19.76 ± 0.09	18.94 ± 0.27	19.08 ± 0.51
Fatty acid classes[*] (% of total FAMEs)			
C10:0	tr	0.36 ± 0.03	0.74 ± 0.04
C12:0	0.12 ± 0.05	6.49 ± 0.68	13.05 ± 1.37
C13:0	tr	tr	tr
C14:0	2.89 ± 0.16	4.02 ± 0.31	5.68 ± 1.05
C15:0	0.42 ± 0.09	0.43 ± 0.08	0.54 ± 0.26
C16:0	23.63 ± 1.63	24.11 ± 2.81	21.62 ± 1.21
C16:1n-9	0.11 ± 0.04	0.13 ± 0.05	0.18 ± 0.02
C16:1n-7	3.24 ± 0.36	3.88 ± 0.91	5.25 ± 0.84
C17:0	0.47 ± 0.11	0.48 ± 0.04	0.59 ± 0.13
C18:0	5.57 ± 0.74	5.44 ± 1.11	5.08 ± 0.23
C18:1n-9	26.25 ± 0.55	27.85 ± 1.65	24.97 ± 1.76
C18:1n-7	1.29 ± 0.38	0.99 ± 0.16	0.98 ± 0.31
C18:2n-6	0.11 ± 0.03	tr	tr
C18:3n-3	1.59 ± 0.13	1.13 ± 0.23	0.95 ± 0.35
C20:0	0.38 ± 0.07	0.42 ± 0.14	0.45 ± 0.07
C20:1n-9	0.95 ± 0.35	0.86 ± 0.22	0.94 ± 0.20
C20:2n-6	0.17 ± 0.01	0.10 ± 0.001	tr
C20:3n-6	0.12 ± 0.04	tr	tr
C20:4n-6, ARA	0.73 ± 0.74	0.49 ± 0.13	0.41 ± 0.06
C20:3n-3	0.15 ± 0.07	0.10 ± 0.14	tr
C20:5n-3, EPA	6.85 ± 0.24	4.24 ± 0.49	3.12 ± 0.74
C22:0	0.19 ± 0.03	0.27 ± 0.01	0.36 ± 0.07
C22:1n-9	0.56 ± 0.13	0.39 ± 0.05	0.35 ± 0.08
C22:6n-3, DHA	13.42 ± 0.89	8.24 ± 0.36	5.97 ± 0.72
C24:1n-9	0.47 ± 0.38	0.37 ± 0.08	0.38 ± 0.08
SFA	33.76 ± 0.14	42.13 ± 4.51	48.24 ± 1.67
MUFA	32.87 ± 1.23	34.46 ± 0.61	33.04 ± 1.45
PUFAn-3	22.01 ± 0.45	13.71 ± 0.22	10.11 ± 0.33
PUFAn-6	11.33 ± 2.20	9.68 ± 1.56	8.62 ± 1.50
PUFAn-9	26.50 ± 0.71	28.00 ± 1.41	25.50 ± 2.12
n-3/n-6	1.99 ± 0.43	1.44 ± 0.25	1.19 ± 0.17

[1]Bioceval GmbH & Co. KG Cuxhaven, Germany.

[2] Protein-rich vegetable ingredients: blend of pea protein concentrate (Lombarda trading srl, Casalbuttano & Uniti; CR, Italy) and wheat gluten meal (Sacchetto spa; Torino, Italy) in 1:1 ratio.
[3]SmartBugs srl (Treviso, Italy).
[4]Consorzio Agrario (Pordenone, Italy).
[$]Mineral supplement composition (% mix): $CaHPO_4*2H_2O$, 78.9; MgO, 2.725; KCl, 0.005; NaCl, 17.65; $FeCO_3$, 0.335; $ZnSO_4*H_2O$, 0.197; $MnSO_4*H_2O$, 0.094; $CuSO_4*5H_2O$, 0.027; Na_2SeO_3, 0.067.
#Vitamin supplement composition (% mix): thiamine HCL Vit B1, 0.16; riboflavin Vit B2, 0.39; pyridoxine HCL Vit B6, 0.21; cyanocobalamine B12, 0.21; niacin Vit PP, 2.12; calcium pantotenate, 0.63; folic acid, 0.10; biotin Vit H, 1.05; myoinositol, 3.15; stay C Roche, 4.51; tocoferol Vit E, 3.15; menadione Vit K3, 0.24; Vit A (2500 UI kg^{-1} diet), 0.026; Vit D3 (2400 UI kg^{-1} diet), 0.05; choline chloride, 83.99.
[*]Values reported as mean of triplicate analyses.
tr: fatty acids below of 0.1% of total FAMEs
SFA = C10:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C24:0.
MUFA = C16:1 n-9 + C16:1 n-7 + C18:1 n-9 + C18:1 n-7 + C20:1 n-9 + C22:1 n-9 + C24:1 n-9.
PUFA n-3 = C18:3 n-3 + C20:3 n-3 + C20:5 n-3 + C22:6 n-3.
PUFA n-6 = C18:2 n-6 + C18:3 n-6 + C20:2 n-6 + C20:3 n-6 + C20:4 n-6.

Methods

The methods used in the attached papers are well established and will be not thoroughly described here; for details, readers are referred to the individual papers. Table 7 gives an overview of the relevant references related to the methodologies utilised.

Table 7. Methods used in the papers produced during the three years of the PhD.

Aim	Parameter	Paper	Method
Microbiota	Denaturing gradient gel electrophoresis and downstream elaborations	1	Bruni et al. (2018)
Microbiota	DNA extraction, library preparation, sequencing, bioinformatics	1, 2	Gajardo et al. (2016)
Gene expression	RNA extraction, cDNA synthesis and qPCR	6, 7, 8	Vargas et al. (2018)
Quality: physical analyses	Colour	3, 4, 5, 8	CIE (1976)
Quality: physical analyses	Texture	3, 5	*Instrumental method (texturometer)*
Quality: physical analyses	Water holding capacity, pH	3, 4, 5, 8	Iaconisi et al. (2018)
Quality: chemical analyses	Proximate composition	3, 4, 5, 8	AOAC (2012)
Quality: chemical analyses	Total lipids, fatty acids	3, 4, 5, 8	Morrison and Smith (1964)
Quality: chemical analyses	Cholesterol	5	Secci et al. (2018)
Lipid metabolism	Estimated indices of lipid metabolism	5, 8	Mattioli et al. (2018); Renaville et al. (2013)
Quality: nutritional properties	Nutritional indices	4, 8	Santos-Silva et al. (2002); Ulbricht and Southgate (1991)
Quality: oxidative status	TBARS, ABTS, DPPH, FRAP	4, 5, 8	Mancini et al. (2015); Vyncke (1970)
Quality: chemical analyses	Volatile organic compounds	3, 4	Colzi et al. (2017)
Quality: sensory analysis	Consumer liking test	3	*Blind product test*

qPCR and microbiota data analysis and bioinformatics

To study the gene expression, the primer sequences were designed using either Primer3 software (210 v. 0.4.0) starting from zebrafish sequences available in Zebrafish Information Network (ZFIN) in ***Paper 6***, or the Primer-BLAST tool available in NCBI (http://www.ncbi.nlm.nih.gov/) for rainbow trout in ***Paper 8***. Data were analysed using the iQ5 optical system software version 2.0, including Genex Macro iQ5 Conversion and Genex Macro iQ5 files (Bio-Rad, CA, USA).

The chromatograms of the sequences obtained from denaturing gradient gel electrophoresis (DGGE) bands were edited using Chromas Lite software (Technelysium Pty Ltd.; Tewantin, QLD, Australia) to verify the absence of ambiguous peaks and to convert them to FASTA format. DECIPHER's Find Chimeras web tool (http://www2.decipher.codes/FindChimeras.html) was used to discard chimeras. Representative sequences were deposited in the GenBank database. The Web-based BLASTN tool (http://www.ncbi.nlm.nih.gov/BLAST) was used to find closely related nucleotide sequences, with different sequence similarity thresholds for different taxonomic levels (Webster et al., 2010). Phylogenetic dendrograms were constructed to display the apparent relatedness of the partial 16S rRNA gene sequences of this study to each other and to other sequences of equivalent length recovered from environmental samples and axenic cultures deposited in the GenBank database. Sequence alignment was performed with ClustalX software (Larkin et al., 2007); distance analysis was carried out according to Jukes and Cantor (1969) followed by phylogenetic tree construction using the neighbour-joining algorithm (Saitou and Nei, 1987) by TREECON 1.3b (Van de Peer and De Wachter, 1994). The robustness of associations between samples (nodes) was evaluated by bootstrap analysis with 1000 replicates.

For high-throughput sequencing data, the QIIME2 pipeline was used (Bolyen et al., 2019). In brief, the demultiplexed paired-end reads were trimmed off to remove the primer sequences, truncated at the position where the median of Phred quality score dropped dramatically and denoised following the DADA2 pipeline to infer amplicon sequence variants (ASVs) (Callahan et al., 2016); the raw sequence data from different Miseq runs were fed into the DADA2 pipeline separately to estimate the run-specific error model. Afterwards, the feature table and the representative sequences generated from different runs were merged. The taxonomic assignment was performed by a Naive Bayes classifier trained on the SILVA 132 99% OTUs, which were trimmed to only include the regions of 16S rRNA amplified by the chosen primers (Bokulich et al., 2018). *Taxa* identified as chloroplast or mitochondria were excluded from the samples. The feature table was conservatively filtered to remove ASVs that had no phylum-level taxonomic annotation or appeared in only one sample. Contaminant sequences were removed from the samples based on the criteria suggested by (Davis et al., 2017), i.e., taking in consideration that contaminants are often found in negative controls and inversely correlate with sample DNA concentration. The ASVs filtered from the raw ASV table were also removed from the representative sequences, which were then aligned by the MAFFT (Katoh and Standley, 2013). A phylogenetic

tree was built from the masked alignment using FastTree (Price et al., 2010). The taxonomy, the core microbiota and the alpha-diversity indices were computed using the ASV table collapsed at the species level. The core microbiota was calculated based on the 80% prevalence threshold and visualized by a Venn's diagram. Faith's phylogenetic diversity (PD), observed OTUs, Shannon's index and Pielou's evenness were computed for the alpha-diversity estimation. The dissimilarity between microbial communities was measured by Jaccard distance, unweighted UniFrac distance, Aitchison distance and PHILR transformed Euclidean distance (Lozupone and Knight, 2005), visualised in EMPeror via principal coordinates analysis (PCoA) (Vázquez-Baeza et al., 2013), and tested for significant difference using permutational multivariate analysis of variance (PERMANOVA, 999 times of permutations) (Anderson, 2001). Finally, significant associations between microbial clades and metadata of interest where tested using MaAsLin2 (version 0.99.12) (https://huttenhower.sph.harvard.edu/maaslin2).

Raw sequence data and sample metadata will be made available on the NCBI SRA database (https://www.ncbi.nlm.nih.gov/sra). Codes used for sequence processing and data analysis will be deposited at a Github repository and will be archived at Zenodo (https://zenodo.org/).

Statistics

Real-time PCR data were analysed either by a two-way ANOVA (***Paper 6***) with both diet and days-post-spawning as the explanatory variables, or by a one-way ANOVA (***Paper 8***).

Data on biometrics, physical traits, proximate and chemical composition, oxidative status, nutritional indices and products:precursors ratios were firstly assessed for normality and homoscedasticity. If normality or homoscedasticity were not met, a boxcox transformation was performed. Successively, a one-way ANOVA followed by a Tukey's test were performed; significance was set at $p=0.05$. Consumer test data were also analysed by a one-way ANOVA, then, penalty analysis was performed on appropriateness data to evaluate the decreases in the overall liking due to specific sensory attributes; Dunnet *post hoc* test was used to identify the significantly different ($p\leq0.05$) liking between the subjects who considered as appropriate a characteristic and those who did not.

Volatile organic compound data were displayed on a 2d plot with a principal component analysis (PCA) procedure.

The software utilised for the statistical analyses in the different Papers are detailed in the annexed Papers.

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Papers 1 to 8

Paper 1

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Characterisation of the intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) fed with *Hermetia illucens* (black soldier fly) partially defatted larva meal as partial dietary protein source

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ABSTRACT

Scientific research has examined the possibility of replacing fishmeal with alternative protein sources in feed for fish. The literature indicates that insects are an eco-friendly nutrient-rich alternative to fishmeal. The purpose of this study was to investigate the effects of including insects in a diet for rainbow trout (*Oncorhynchus mykiss*), by analysing organosomatic parameters, fillet yield and intestinal bacterial communities. Three experimental diets were formulated: a control diet with fishmeal as main protein source, and diets Hi25 and Hi50 where 25% and 50% of fishmeal, respectively, was replaced with partially defatted meal of larvae of *Hermetia illucens* (Hi; Diptera: Stratiomydae). At the end of the trial, organosomatic parameters and fillet yield were recorded. To profile the complex intestinal mucosa- and digesta-associated bacterial communities (MAB and DAB), denaturing gradient gel electrophoresis was performed on bacterial DNA extracted from intestinal mucosa and digesta, followed by sequencing of selected bands. Rainbow trout fed the alternative diets showed the same organosomatic indices and fillet yields as the control group, but increased bacterial community biodiversity, structure and composition, with ANOSIM $p < .05$ and $p < .01$ for MAB and DAB, respectively. The sequencing highlighted a clear prevalence of γ-Proteobacteria in all samples, though α- and β-Proteobacteria and Actinobacteria were also present in MAB of insect-fed fish; DAB of insect-fed fish showed a clear increase in the Firmicutes phylum compared to the control group. The results suggest that *H. illucens* partially defatted larva meal is a valid alternative protein source and can replace up to 50% of fishmeal in rainbow trout feed without impairing organosomatic indices nor fillet yield. The microbiological assays revealed that the intestinal bacterial communities were sensitive to dietary changes, showing modified community structure and increased biodiversity in the Hi-fed groups. We discuss the effects that modified bacterial communities could have on fish biology. There is a good possibility of further studies on the functional role of bacteria.

1. Introduction

Using insect meal instead of fishmeal is becoming more common in the aquaculture sector of many countries. Not only is fishmeal not eco-friendly as principal dietary protein source, but it is also becoming costlier. Issues such as the increasing global demand for fish protein, the impact of fishmeal production on the ecology of fishing grounds, its shortage and its high price have brought attention to the need for alternative dietary protein sources (FAO, 2016, 2014). Animal and fishery by-products as well as plant-derived material are now used as substitutes (FAO, 2014). Regrettably, plant protein derivatives rarely have a balanced essential amino acid (EAA) profile and often contain antinutritive factors (Oliva-Teles et al., 2015). Processed animal protein is considered a valuable alternative as it has a better EAA profile and is more digestible than plant proteins; nevertheless, within the Europe Community, restrictions on the use of certain processed animal proteins

Abbreviations: DAB, digesta-associated bacterial community; DISAFA, Department of Agricultural, Forest and Food Sciences; DISPAA, Department of Agri-Food Production and Environmental Sciences; EFSA, European Food and Safety Authority; Hi, *Hermetia illucens*; LAB, lactic acid bacteria; MAB, mucosa-associated bacterial community; OTU, operational taxonomic unit

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Full title: Characterisation of the intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) fed with *Hermetia illucens* (black soldier fly) partially defatted larva meal as partial dietary protein source

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Abstract

Scientific research has examined the possibility of replacing fishmeal with alternative protein sources in feed for fish. The literature indicates that insects are an eco-friendly nutrient-rich alternative to fishmeal. The purpose of this study was to investigate the effects of including insects in a diet for rainbow trout (Oncorhynchus mykiss), by analysing organosomatic parameters, fillet yield and intestinal bacterial communities. Three experimental diets were formulated: a control diet with fishmeal as main protein source, and diets Hi25 and Hi50 where 25% and 50% of fishmeal, respectively, was replaced with partially defatted meal of larvae of Hermetia illucens (Hi; Diptera: Stratiomydae). At the end of the trial, organosomatic parameters and fillet yield were recorded. To profile the complex intestinal mucosa- and digesta-associated bacterial communities (MAB and DAB), denaturing gradient gel electrophoresis was performed on bacterial DNA extracted from intestinal mucosa and digesta, followed by sequencing of selected bands. Rainbow trout fed the alternative diets showed the same organosomatic indices and fillet yields as the control group, but increased bacterial community biodiversity, structure and composition, with ANOSIM $p<0.05$ and $p<0.01$ for MAB

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Key words: rainbow trout, insect, black soldier fly, intestinal microbial community, DGGE, sustainable aquaculture.

Abbreviations

DAB digesta-associated bacterial community
DISAFA Department of Agricultural, Forest and Food Sciences
DISPAA Department of Agri-Food Production and Environmental Sciences
EFSA European Food and Safety Authority
Hi *Hermetia illucens*
LAB lactic acid bacteria
MAB mucosa-associated bacterial community
OTU operational taxonomic unit

1. Introduction

Using insect meal instead of fishmeal is becoming more common in the aquaculture sector of many countries. Not only is fishmeal not eco-friendly as principal dietary protein source, but it is also becoming costlier. Issues such as the increasing global demand for fish protein, the impact of fishmeal production on the ecology of fishing grounds, its shortage and its high price have brought attention to the need for alternative dietary protein sources (FAO, 2016, 2014). Animal and fishery by-products as well as plant-derived material are now used as substitutes (FAO, 2014). Regrettably, plant protein derivatives rarely have a balanced essential amino acid (EAA) profile and often contain antinutritive factors (Oliva-Teles et al., 2015). Processed animal protein is considered a valuable alternative as it has a better EAA profile and is more digestible than plant proteins; nevertheless, within the Europe Community, restrictions on the use of certain processed animal proteins persist as protection against transmissible spongiform encephalopathies (Regulation 68/2013/EC, 2013). Insects have recently attracted increasing attention as a sustainable nutrient source for feed, not only in Europe but also around the world. Indeed, insects are a good source of EAA, lipids,

vitamins and minerals (Henry et al., 2015; van Huis et al., 2013); they grow and reproduce quickly and easily on low-quality organic waste and manure (van Huis et al., 2013); they have a small ecological footprint and high feed conversion efficiency (Makkar et al., 2014), and can reasonably foster a circular bioeconomy. Finally, the use of processed insects in feed for aquaculture animals was recently allowed by the European Commission (Regulation 2017/893/EC, 2017).

Compared to other insects, the Diptera order and in particular the species Hermetia illucens (Hi), also known as black soldier fly, show an EAA pattern very similar to fishmeal (Henry et al., 2015) and are therefore a good alternative protein source. Recent trials substituting up to 40% of fishmeal with Hi meal have shown that it effectively supports fish growth (Lock et al., 2016; Magalhães et al., 2017; Renna et al., 2017). Regarding the hygienic concerns of the European Food and Safety Authority (EFSA) about insect rearing and characteristics (EFSA Scientific Committee, 2015), adults of Hi do not feed, thus diminishing the likelihood of disease transmission to their progeny (Makkar et al., 2014). Insects are rich in chitin, which reduces their digestibility but, as underlined by Karlsen et al. (2017), dietary chitin may select a beneficial intestinal microbiota.

Different diets affect the structure and the composition of fish intestinal bacterial communities in different ways (Hartviksen et al., 2014; Wang et al., 2017; Zhou et al., 2013); the communities in turn affect the digestive functions and immune responses of the host (Ghanbari et al., 2015; Lyons et al., 2017a). The impact of bacteria on digestive functions can be summarised as providing essential nutrient and non-nutrient factors and as increasing the host's ability to harvest nutrients from feed, for instance by producing digestive enzymes that break down chitin or cellulose (Gomez et al., 2013; Ray et al., 2012; Ringø et al., 1995). Additionally, bacteria dwelling in the gastrointestinal tract co-exist in dynamic equilibrium with occasional pathogens; indeed, gut-associated lymphoid tissue sorts microorganisms and responds either with tolerance or an immune response (Merrifield et al., 2010; Nayak, 2010; Pérez et al., 2010; Ringø et al., 2010a). If the composition of the intestinal bacterial community is known and the principles of its assembly and preservation are understood, it can be manipulated to improve the health of the host (Merrifield et al., 2010; Ringø et al., 2010b; Roeselers et al., 2011; van Kessel et al., 2011). The intestinal bacterial community can be considered a key to healthy fish and productive aquaculture plants.

The mucosa-associated bacterial community (MAB) could have a greater impact than the digesta-associated community (DAB) on the biology of the host. It is therefore worthwhile making an effort to examine the MAB. To the authors' knowledge, this is the first study aimed at assessing the changes in rainbow trout MAB and DAB induced by dietary insect meal. Denaturing gradient gel electrophoresis (DGGE) and band sequencing were used to acquire an overview of these complex communities, to identify dominant bacterial groups, and to glean insights into the effect of the sequenced bacterial strains on fish.

2. Materials and methods

2.1. Experimental diets and growth

The experimental protocol applied in this study was designed according to the ethical standards approved by the current European Directive 2010/63/EU on the protection of animals used for scientific purposes.

The samples analysed in this study were retrieved from the growth trial described in Renna et al. (2017) using meal of partially defatted Hi larvae reared on vegetable by-product substrate purchased from Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, 141 Germany). The Hi larva meal had the following chemical composition: dry matter (DM): 94.18%; crude protein (CP): 55.34 g/100 g DM; ether extract (EE): 17.97 g/100 g DM; ash: 7.12 g/100 g DM; chitin: 5.00 g/100 g DM; nitrogen free extracts (NFE): 14.57 g/100 g DM. It had a gross energy (GE) of 24.37 MJ/kg DM. Three experimental diets were formulated with increasing percentages of Hi larva meal: one control diet (Hi0) and two test diets (Hi25 and Hi50). The diets were formulated to be isonitrogenous (CP: about 45 g/100 g DM), isolipidic (EE: about 15 g/100 g DM), and isoenergetic (GE: about 22 MJ/kg DM). Hi0 contained fishmeal (600 g/kg) as the main protein source, whereas Hi25 and Hi50 had 25% and 50% of the fishmeal replaced by Hi meal, respectively (a total of 20% and 40% of Hi meal in Hi25 and Hi50, respectively).

Rainbow trout (*Oncorhynchus mykiss*) of 178.9 ± 9.81 g initial body weight were fed the experimental diets for 78 days (Renna et al., 2017).

Table 1 shows the ingredients, the chemical composition of the experimental diets containing Hermetia illucens larva meal and the main trout growth performance measures as reported in Renna et al. (2017).

2.2. Sampling, fillet yield and organosomatic indices

At the end of the feeding trial followed by one day of starvation, 90 fish (30 per group) were anesthetised with 60 mg/L MS222, killed and immediately transported on ice to the Department of Agri-Food Production and Environmental Sciences (DISPAA), University of Florence (Florence, Italy), where each was weighed and dissected. The fillets (from 90 fish), 30 livers (10 samples per group) and 18 viscera (6 samples per group) were individually weighed to assess fillet yield (FY=weight of fillets with skin×100/BW), visceral index (VSI=total viscera weight×100/BW) and hepatosomatic index (HSI=liver weight×100/BW).

2.3. Sampling for microbiological analysis

On arrival at DISPAA, the abdomen of 18 fish (6 samples per group) was cut open with alcohol-disinfected tools and the intestine, from just after the pyloric caeca to the anus, was removed and placed on tinfoil. Perivisceral fat was removed and the digesta were gently squeezed out into a tube. The intestine was then cut longitudinally, rinsed by flushing in sterile phosphate-buffered saline to remove faecal traces and placed in a tube. Pyloric caeca were not analysed due to the limited resources allocated to the present study and because this segment

presents the highest technical difficulties, as stated in the literature (Gajardo et al., 2016). We collected: 6 samples of intestinal tissue for each of the three groups to investigate the mucosa-associated bacterial community (MAB) (18 tubes); 6 samples of digesta for each of the three groups to investigate digesta-associated bacterial community (DAB) (18 tubes). The 36 tubes were stored at -80 °C until microbiological analysis.

2.4. DGGE analyses

The 18 intestine-tissue and 18 digesta samples were processed at the Research Centre for Agriculture and Environment (CREA-AA, Florence, Italy) to assess the biodiversity and composition of MAB and DAB. Each intestine sample was thawed on ice, homogenised with an UltraTurrax® T 25 (IKA-Labor Technik, Staufen, Germany) at 20,500 rpm for 1.5 minutes in an ice bath and immediately processed for total DNA extraction. The DNA of intestinal homogenates and digesta was extracted with the QIAamp®DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the protocol for isolation of DNA from stools for pathogen detection, with minor changes: a) the volume of elution Buffer AE was decreased to 75 µL to obtain a higher concentration of DNA in the final eluate; b) the incubation time of Buffer AE was increased to 5 minutes to increase DNA yield.

The V6-V8 region of the template DNA was amplified with the primer set GC-986F (5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TA -3') and 1401R (5'- CGG TGT GTA CAA GAC CC -3'), as designed by Nübel et al. (1996). This set of primers proved to function well and homogeneously among all samples, as showed in the DGGE profile of bacterial communities (S1 and S2 Figures). Amplification reactions were carried out in a T100™ Thermal Cycler (Bio-Rad) in 25 µL of a mixture containing: 2 µL template DNA, 1× Green GoTaq® Flexi Buffer (Promega Corporation, Madison, WI, USA), 1.5 mmol L-1 $MgCl_2$ (Promega), 200 µmol L-1 dNTPs (Promega), 10 pmol of each primer and 1U GoTaq® G2 Flexi DNA Polymerase (Promega), under reaction conditions of: denaturation at 95 °C for 2 minutes, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 45 s and final elongation at 72 °C for 5 minutes. The amplicons from several amplification reactions were pooled to minimise PCR biases. Amplicon yield was estimated by comparison with the Low DNA mass ladder (Invitrogen, Carlsbad, CA, USA) using a Chemidoc Apparatus (Bio-Rad Laboratories Inc., Hercules, CA, USA).

DGGEs were performed on a DCode™ Universal Mutation Detection System (Bio-Rad), loading 600 ng of the amplicons onto a 6% polyacrylamide gel (acrylamide/bis 37.5:1; EuroClone S.p.A, Pero, Milan, Italy), with a linear denaturing gradient from 45% to 65% (v/v) obtained from a 100% denaturing solution containing 40% formamide (v/v) (Sigma-Aldrich GmbH, St. Louis, MO, USA) and 7 M urea (Promega). The gels were run for 17 h in 1×TAE buffer at constant voltage (80 V) and temperature (60 °C). At the end, the gels were

stained with SYBR® GOLD (Molecular Probes, Eugene, OR, USA) diluted 1:10,000 in 1×TAE buffer. Gel images were digitalised using the Chemidoc apparatus and optimised for analysis by enhancing contrast and greyscale.

2.5. Sequencing

The middle portion of 38 selected DGGE bands was excised and placed in 30 µL distilled water. Given the greater interest in MAB, more bands were sequenced from intestinal than from digesta samples. The PCR products were eluted through freezing and thawing (Throbäck et al., 2004) and reamplified using the primer pairs described above. Amplicons were checked by DGGE for the presence of a single band and then directly sequenced by Macrogen Service (Macrogen Ltd., Amsterdam, The Netherlands, http://www.macrogen.com).

The sequence chromatograms were edited using Chromas Lite software (v2.1.1; Technelysium Pty Ltd; Tewantin, QLD, Australia; http://www.technelysium.com.au/chromas_lite.htm) to verify the absence of ambiguous peaks and to convert them to FASTA format. DECIPHER's Find Chimeras web tool (http://decipher.cee.wisc.edu) was used to uncover chimeras in the 16S rDNA sequences. Representative sequences were deposited in the GenBank database under accession numbers KY270784-KY270810. The Web-based BLASTN tool (http://www.ncbi.nlm.nih.gov/BLAST) was used to find closely related nucleotide sequences. To increase the accuracy of the assignments, different sequence similarity thresholds were used for different taxonomic levels: a similarity ≥97% for species level identification and 95%, 90%, 85%, 80% and 75% for assignment at genus, family, order, class and phylum level, respectively (Webster et al., 2010). Phylogenetic dendrograms were constructed to display the apparent relatedness of the partial 16S rRNA gene sequences of this study to each other and to other sequences of equivalent length recovered from environmental samples and axenic cultures deposited in the GenBank database. Sequence alignment was performed with ClustalX 2.0.11 software (Larkin et al., 2007); distance analysis was carried out according to Jukes and Cantor (1969) followed by phylogenetic tree construction using the neighbour-joining algorithm (Saitou and Nei, 1987) by TREECON 1.3b (Van de Peer and De Wachter, 1994). The robustness of associations between samples (nodes) was evaluated by bootstrap analysis with 1000 replicates.

2.6. Statistical analysis

Data on fillet yield and organosomatic indices was checked for normality and homoscedasticity, then one-way ANOVA was performed with the diet as independent variable, using the Paleontological Statistics Software Package (PAST; Hammer et al., 2001).

The DGGE banding patterns were normalised and analysed using GelCompar II software v 4.6 (Applied Maths, Sint-Martens-Latem, Belgium). Although amplification products from different bacterial groups can co-migrate, each band

was considered to match a single bacterial species or group, and band intensity (the relative surface of the peak compared to the surfaces of all the peaks in the profile) was considered to indicate the relative abundance of the corresponding species or group (Fromin et al., 2002). Bands shared by samples were classified as the same band when they were within a 0.8% range of total pattern length from each other. Phylotype richness (number of DGGE bands), Shannon-Wiener diversity index (H'=-$\Sigma R \times pi \times \ln pi$, where R is the number of phylotypes/bands in the sample and pi is the relative intensity of the ith phylotype/band) and Simpson's evenness index ($\lambda = \Sigma R \times pi2$) were calculated with GelCompar II. Simpson data were log-transformed to fit normality. One-way ANOVA followed by a Tukey HSD post hoc test was performed on the results of the three indices using PAST.

The banding patterns of the DGGEs were extracted as band-intensity matching tables, then normalised by calculating the relative intensity of each band (ratio of the intensity of each band divided by the sum of the intensities of all bands in the same lane) and finally imported into PAST for further multivariate analyses. Non-metric multidimensional scaling (nMDS) was used to represent the distance between each sample in two-dimensional space. One-way analysis of similarity (ANOSIM) followed by pairwise comparisons was conducted to determine the extent of differences in MAB and DAB between the three dietary groups. ANOSIM and nMDS were performed using the Bray-Curtis distance measure and 9,999 permutational tests; the accuracy of the nMDS plots was determined by calculating a 2D stress value.

3. Results

3.1. Fillet yield and organosomatic indices

VSI did not highlight differences among treatments and ranged from 7.43 to 7.91 for Hi25 and Hi50, respectively (p-value: 0.50). Similarly, no differences were reported for HSI (1.48, 1.53 and 1.53 for Hio, Hi25 and Hi50, respectively; p-value: 0.76). As far as fillet yield is concerned, values were above 60% and without differences among treatments (Hio: 62.4; Hi25: 63.1; Hi50: 61.8; p-value: 0.57)

3.2. Intestinal bacterial communities

The DGGE banding patterns were analysed using biodiversity indices (Table 2). No significant differences were found among the DAB samples; by contrast, the biodiversity of the MAB Hi25 group was significantly higher than the control group ($p<0.05$) and that of the Hi50 group was intermediate between the two; the SEM of richness and Shannon index values seemed higher in the Hi25 and Hi50 groups.

The intensity of staining showed a decreasing trend from the Hio to Hi50 groups, especially in MAB samples (S1 Figure).

The nMDS plots showed slight clustering in relation to diet in MAB and DAB samples (Figure 1). In both, the Hi25 samples seemed to form a loose yet separate cluster from the control. The relative positions of the points in the plots

was moderately reliable in the MAB (stress=0.1944) and DAB (stress=0.2271) samples.

Again, in the case of MAB and DAB samples, ANOSIM tests (Table 3) revealed significant differences between groups ($p<0.05$ and $p<0.01$, respectively). The results of pairwise comparisons are summarised in Table 4, where differences between all groups were significant ($p<0.01$) in DAB samples, whereas in MAB samples, only the pair Hio-Hi25 displayed a significant difference ($p<0.05$).

3.3. Sequencing

A total of 29 and 19 bands were retrieved from the MAB and DAB DGGE gels, respectively; of these, 20 and 14 bands from MAB and DAB, respectively, were successfully sequenced; 27 sequences were deposited in the GenBank database. Operational taxonomic units (OTUs) were identified at species level in 24/27, at *genus* level in 2/27 and at family level in 1/27 sequences (Tables 5 and 6). The OTUs proved related to the *phyla* Proteobacteria, Firmicutes and Actinobacteria, including eleven *genera*: *Acinetobacter*, *Aeromonas*, *Brevundimonas*, *Carnobacterium*, *Citrobacter*, *Curtobacterium*, *Delftia*, *Kluyvera*, *Pseudomonas*, *Shewanella* and *Staphylococcus*.

Bands in different lanes at the same height were considered to belong to the same or to a very close phylogenetic bacterial strain. The OTUs from MAB samples were related to eight *genera* in the Proteobacteria and Actinobacteria *phyla*: *Curtobacterium* (Actinobacteria); *Brevundimonas* (α-Proteobacteria); *Delftia* (β-Proteobacteria); *Acinetobacter*, *Aeromonas*, *Citrobacter*, *Pseudomonas* and *Shewanella* (γ-Proteobacteria). The core set of OTUs resistant to dietary changes in MAB samples was related to *Pseudomonas* spp. and *Shewanella* spp. OTUs related to *Aeromonas rivipollensis* were only abundant in the control group, while the insect-fed groups were rich in bands related to *Citrobacter gillenii*, *Pseudomonas* spp. and *Delftia acidovorans*; bands related to *Acinetobacter* spp., *Brevundimonas* spp., *Curtobacterium flaccumfaciens* and *Delftia acidovorans* were only recovered from insect-fed fish and were sporadic. The OTUs from DAB samples were related to genera in the Proteobacteria and Firmicutes *phyla*: *Carnobacterium* and *Staphylococcus* (Bacilli); *Aeromonas*, *Kluyvera* and *Shewanella* (γ-Proteobacteria). The core set of OTUs resistant to dietary changes in DAB samples was linked to *Shewanella schlegeliana* and *Aeromonas rivipollensis*. OTUs related to *Citrobacter gillenii* and *Kluyvera intermedia* (both belonging to the family Enterobacteriaceae) and *Carnobacterium divergens* were abundant in the insect-fed groups. Control MAB and DAB samples showed very similar DGGE banding patterns, which were more variable in the insect-fed groups.

4. Discussion

The main results obtained by Renna et al. (2017) indicated that substituting up to 50% of fishmeal with a partially defatted Hi larva meal in diets for rainbow

trout did not affect survival or growth performance. Differences in apparent digestibility were only found between the Hi25 and Hi50 groups, with the control showing intermediate values. The present study reported that fillet yield and organosomatic performance were not statistically different between control-fed and insect-fed fish. On the other hand, as discussed in detail by Renna et al. (2017), feeding salmonid species with H. illucens could negatively affect growth performance (St-Hilaire et al., 2007; Stamer et al., 2014), but the outcome depended on the insect's life stage (larvae vs. prepupae), fat content and rearing substrate (Sealey et al., 2011), on the processing techniques used (Lock et al., 2016) and on the fish life stage. As suggested by Magalhães et al. (2017), detailed information concerning substrate, handling and processing of the insect as well as the composition of each batch of insect meal is needed in order to explain the performance results. Nevertheless, as reported by Oliva-Teles et al. (2015), aquaculture outcome is not defined solely by fish performance, but issues concerning fish welfare also need to be taken into account. These include immunological status, oxidative status and the influence of diets on intestinal microbiota, which directly affect the digestive functions and immune response of the host (Ghanbari et al., 2015; Lyons et al., 2017a) and are therefore considered a key to healthy fish and productive aquaculture plants.

The results of studies on salmonids fed diets containing chitin from various sources are inconsistent. Indeed, chitin is suspected to diminish feed availability and digestibility in fish (Kroeckel et al., 2012; Olsen et al., 2006). Krill meal seemed to depress growth performance of *Oncorhynchus keta* fingerlings (Murai et al., 1980) and rainbow trout (Wojno and Dabrowska, 1984), and chitin sourced from shrimp (*Pandalus borealis*), included in the diet at a concentration of 2% and 5%, seemed to depress Atlantic salmon performance (Karlsen et al., 2017). However, the results of the present study were positive despite the presence of chitin in the Hi feeds. Moreover, Lellis and Barrows (2000) reported that 6% chitin supplementation enhanced growth in rainbow trout juveniles, and Atlantic salmon, fed a diet in which 50% of fishmeal had been replaced by northern krill (*Meganyctiphanes norvegica*) meal, showed no differences in terms of growth performance (Ringø et al., 2006a). A reason for these contrasting results could be that under certain conditions chitin may counterbalance the negative effects that it itself produces. In fact, some authors have suggested that chitin may be targeted by a positive intestinal microbial community, which improves host organism performance and health status (Karlsen et al., 2017; Ringø et al., 2006a).

Bearing in mind that DGGE only detects dominant bacterial groups (Muyzer et al., 1993), the present study indicates that the fish microbial community is plastic and can be manipulated by addition of insect meal to feed, in line with the majority of studies on other dietary sources (Desai et al., 2012; Hartviksen et al., 2014; Ingerslev et al., 2014; Navarrete et al., 2012; Ringø et al., 2006a; Zhou et al., 2013). Biodiversity parameters of MAB and DAB were increased by dietary administration of the insect meal, in general agreement with studies assessing

the effect of dietary krill or inclusion of 5-20% chitin in the diet of salmonids (Askarian et al., 2012; Ringø et al., 2012) but in contrast with studies on the effect of plant proteins (Bakke-McKellep et al., 2007; Reveco et al., 2014). The MAB control group of the present study showed the lowest richness and diversity values, the Hi25 group had the highest while the Hi50 stayed in an intermediate position, in contrast with the hypothesis that a higher content of Hi meal in the diet is accompanied by greater changes in the intestinal microbial community. It is possible that the weak staining of the MAB Hi50 samples was due to less abundant taxa, leading to underestimation of subtle composition variations: hence, the actual diversity could be higher than expressed by the diversity indices. It is commonly recognised that high microbial diversity can compete with pathogens for nutrients and colonization sites (Cerezuela et al., 2013) and that it bestows resilience, a notion Yachi and Loreau (1999) referred to as the 'Insurance hypothesis'. This concept could explain the similarly good performance recorded by fish fed control and insect diets, although the chitin could have decreased nutrient digestibility.

The diversity indices of the present study for insect-fed trout generally showed a more variable response than for control groups, as indicated by SEM; nMDS plots indicated the same trend. Ordination methods are a quick way to make a 2D plot of trends between groups when many variables are involved, though they unfortunately do not quantify significance that would enable scientists to interpret the plots (Al-Hisnawi et al., 2015; Dimitroglou et al., 2009; Forberg et al., 2016; Heikkinen et al., 2006; Ingerslev et al., 2014). Multivariate tests, an established tool in other disciplines dealing with complex microbial communities and now also used in studies assessing fish gastrointestinal communities (Zhou et al., 2014), back up the results of biodiversity indices and nMDS plots (De Mesel et al., 2004; Lagomarsino et al., 2016; Lam et al., 2008). The ANOSIM tests of the present study showed that most bacterial community structures were diet specific. Since the high within-group variability indicated the influence of latent factors, experiments including more factors (e.g. farming management, tank and genotype) are required to discriminate the different sources of variation. Similarly, although pooling within-group replicates is a common practice in studies of fish intestinal microbial community (Dimitroglou et al., 2009; Hartviksen et al., 2014; Navarrete et al., 2009; Zhou et al., 2009), it obscures interindividual variability, as indicated by several authors (Desai et al., 2012; Reveco et al., 2014; Ringø et al., 2006b; Yang et al., 2012). Indeed, Navarrete et al. (2012) posited that different host genetics shapes a unique niche that singles out a specific bacterial community. The question of how the host background shapes the community is already on the agenda in other fields of research (Archie and Theis, 2011; McKnite et al., 2012; Spor et al., 2011) and should also be addressed in aquaculture.

Our sequencing results were in line with the general consensus on rainbow trout intestinal bacterial composition, which is principally composed of γ-Proteobacteria and Firmicutes (Desai et al., 2012; Lyons et al., 2017b; Mansfield

et al., 2010; Navarrete et al., 2010; Spanggaard et al., 2000). The present study seems to be the first to find the *Acinetobacter*, *Brevundimonas* and *Shewanella* genera in rainbow trout MAB. In addition, MAB composition differed from DAB, in line with other studies in the literature (Gajardo et al., 2016; Hartviksen et al., 2014; Merrifield et al., 2009a, 2009b). It therefore seems worthwhile analysing the two substrates separately. For instance, in MAB, but not in DAB, of insect-fed fish, α- and β-Proteobacteria and Actinobacteria were also found, coherent with Lyons et al. (2017b). To sum up, like other studies in the literature (Lyons et al., 2017b; Roeselers et al., 2011; van Kessel et al., 2011; Xia et al., 2014), we observed few abundant genera that dominated the community pattern.

Lyons et al. (2017a) indicated that rainbow trout intestinal bacteria could contain genes that could positively influence the host's digestive metabolism. In the present study, OTUs attributable to lactic acid bacteria (LAB, Firmicutes) were only found in DAB samples from insect-fed fish, in contrast with the literature on salmonids (Al-Hisnawi et al., 2015; Askarian et al., 2012; Bakke-McKellep et al., 2007; Lyons et al., 2017b; Merrifield et al., 2009b). Making a hypothesis similar to that of Gajardo et al. (2016), we suggest that chitin was the preferential growth substrate for LAB, which could increase the digestibility of fibre, as found for soybean-based diets (Desai et al., 2012). Several other OTUs retrieved in this study could account for effects on fish physiology. This is why we propose an overview of these hypothetical effects, while bearing in mind that the mere presence of a microbe in the intestine does not necessarily imply a functional role (Zhou et al., 2013).

Shewanella spp. were clearly ubiquitous, especially *S. schlegeliana*. Described by Satomi et al. (2003) for the first time, strains of *S. schlegeliana* have interesting enzyme activities and, above all, their fatty acids contain 18.6% eicosapentaenoic acid. We assume that *S. schlegeliana* contributed to the nutrition of the present rainbow trout. On the other hand, this species has also been found to produce trimethylamine (Satomi et al., 2003), indicating that further studies on the interaction between this species and the host are necessary. *Pseudomonas stutzeri* was part of the core set of OTUs of MAB, with the insect-fed groups particularly rich in these bacteria. *Pseudomonas* spp. are listed as probiotics by Nayak (2010), have antiviral activity (reviewed by Balcázar et al., 2006), together with *Staphylococcus* spp. may promote nutritional processes in Arctic charr (*Salvelinus alpinus* L.) (Ringø et al., 1995) and Askarian et al. (2012) isolated a *Pseudomonas* sp. strain with amylase, cellulase and lipase activity from the gastrointestinal tract of Atlantic salmon fed a control diet. *Acinetobacter radioresistens* was found in rainbow trout MAB for the first time by the present study; it conceivably played an important role in nutrient digestion since Acinetobacter spp. extracted from Atlantic salmon displayed chitinase, amylase, cellulase and phytase activity and seemed to inhibit the growth of the pathogens *Vibrio anguillarum* and *Moritella viscosa* (Askarian et al., 2012). *Aeromonas* spp. strains, observed in the control group MAB and DAB of the present study, are

listed as probiotics by Nayak (2010) and known for their cellulase activity (Li et al., 2014). *Carnobacterium divergens* was abundant in DAB samples of the insect-fed groups. Bacteria of the *Carnobacterium genus* are well-known probiotics in salmonids and have several functions: in vitro growth inhibition of pathogens, stimulation of non-specific immune response and *in vivo* improvement of disease resistance. Together with *Staphylococcus* species, they may also improve the digestion of proteins and carbohydrates (Al-Hisnawi et al., 2015; Askarian et al., 2012; Balcázar et al., 2006; Mansfield et al., 2010; Ringø et al., 1995, 2010a).

5. Conclusions

Our results indicate that partially defatted *H. illucens* larva meal, a valid alternative protein source to fishmeal for feeding rainbow trout as reported by previous studies, sensitively changed mucosa- and digesta-associated intestinal bacteria communities, showing higher biodiversity in the insect-fed groups. Some bacteria may protect fish from pathogens as well as enhancing digestion, physiological functions and welfare in general. The present results may be useful for future research into salmonid microbial communities and into interactions between host, guest and diet. To delve into the biological consequences of the host-guest interaction, we suggest combining description of bacterial communities with functional methods, such as metagenomics, metabolomics and challenge tests.

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Table 1. Ingredients (% fresh matter), chemical composition (g/100 g DM) of experimental diets containing *Hermetia illucens* (Hi) larvae meal and major performances of trout fed diets (from Renna et al., 2017).

	Hi0	**Hi25**	**Hi50**	**p-value**
Ingredients				
FM	60	45	30	
Hi meal	0	20	40	
Wheat meal	4	4	4	
Wheat bran	9	6	3	
Starch (D500)	15	15	15	
Fish oil	9	7	5	
Vitamin mixture	1.5	1.5	1.5	
Mineral mixture	1.5	1.5	1.5	
Chemical composition				
Dry matter	96.07	94.93	95.63	
Crude protein	45.20	44.86	45.00	
Ether extract	15.86	15.74	15.81	
Ash	11.40	11.43	10.11	
Chitin	0	1.05	2.09	
Nitrogen free extract	27.54	26.92	26.99	
Growth performances (n = 4)				
Weight gain	360.5	366.5	358.9	0.840
Feed conversion ratio	0.90	0.88	0.90	0.739

Table 2. Values of the biodiversity parameters for the mucosa- and digesta-associated bacterial community (MAB and DAB, respectively) (value ± SEM).

	M A B		
	Hio	**Hi25**	**Hi50**
Richness	6.83 ± 0.87^{a}	14.83 ± 2.12^{b}	9.50 ± 1.98^{ab}
Shannon	1.87 ± 0.12^{a}	2.63 ± 0.14^{b}	2.11 ± 0.23^{ab}
Simpson	0.16 ± 0.02^{a}	0.08 ± 0.01^{b}	0.14 ± 0.03^{ab}
	D A B		
	Hio	**Hi25**	**Hi50**
Richness	7.50 ± 0.89	11.00 ± 1.34	9.50 ± 1.91
Shannon	1.96 ± 0.12	2.32 ± 0.14	2.14 ± 0.17
Simpson	0.15 ± 0.02	0.11 ± 0.02	0.13 ± 0.02

Different superscripts indicate significant differences ($p<0.05$).

Table 3. Global ANOSIM R and p-values of the mucosa- and digesta-associated bacterial community (MAB and DAB, respectively).

	MAB	**DAB**
ANOSIM R value	0.2084*	0.4490**

*** p-value<0.01; * p-value<0.05.*

Table 4. ANOSIM results of the pairwise comparisons.

		MAB		DAB	
		Hio	**Hi25**	**Hio**	**Hi25**
ANOSIM R values	**Hi25**	0.3861*		0.3500**	
	Hi50	0.1343	0.1176	0.5222**	0.5074**

** *p-values<0.01; * p-value<0.05.*

Table 5. Bands excised and identified (% similarity to nearest neighbour) from DGGE gels of mucosa-associated intestinal bacteria (MAB). Numbers in the three right-hand columns show the number of samples (from six) per dietary group where a band at the same height of the excised one was found.

DGGE-band code	Isolation source	Accession no.	Nearest BLASTN match (GenBank accession no.; % sequence similarity)	Taxonomic classification
M25	Hi25	KY270803	*Curtobacterium flaccumfaciens* LMG 3645 (NR_025467.1; 100%)	*Curtobacterium flaccumfaciens*
M19	Hi25	KY270799	*Brevundimonas aurantiaca* CB-R (NR_028889.1; 100%)	*Brevundimonas aurantiaca*
M18	Hi25	KY270798	*Brevundimonas subvibrioides* ATCC 15264 (NR_074136.1; 97%)	*Brevundimonas subvibrioides*
M17	Hi25	KY270797	*Delftia acidovorans* NBRC 14950 (NR_113708.1; 99%)	*Delftia acidovorans*
M37b	Hi25	KY270809	*Delftia acidovorans* NBRC 14950 (NR_113708.1; 100%)	*Delftia acidovorans*
M11	Hio	KY270793	Aeromonas rivipollensis P2G1 (NR_144574.1; 100%)	*Aeromonas rivipollensis*
M16	Hi25	KY270796	*Citrobacter gillenii* CDC 4693-86 (NR_041697.1; 99%)	*Citrobacter gillenii*
M15	Hi25	KY270795	*Citrobacter gillenii* CDC 4693-86 (NR_041697.1; 95%)	Unclassified *Citrobacter*
M9	Hio	KY270794	*Pseudomonas stutzeri* ATCC 17588 (NR_103934.1; 99%)	*Pseudomonas stutzeri*
M24a	Hi25	KY270802	*Pseudomonas stutzeri* ATCC 17588 (NR_103934.1; 100%)	*Pseudomonas stutzeri*
M35	Hi50	KY270808	*Pseudomonas stutzeri* ATCC 17588 (NR_103934.1; 99%)	*Pseudomonas stutzeri*
M22	Hi25	KY270801	*Pseudomonas glareae* KMM 9500 (NR_145562.1; 96%)	Unclassified *Pseudomonas*
M29	Hi50	KY270806	*Acinetobacter oleivorans* DR1 (NR_102814.1; 99%)	*Acinetobacter oleivorans*
M20	Hi25	KY270800	*Acinetobacter radioresistens* NBRC (NR_114074.1; 100%)	*Acinetobacter radioresistens*
M12 and M1	Hio	KY270784	*Shewanella schlegeliana* HRKA1 (NR_024792.1; 99%)	*Shewanella schlegeliana*
M26	Hi25	KY270804	*Shewanella schlegeliana* HRKA1 (NR_024792.1; 99%)	*Shewanella schlegeliana*
M38	Hi25	KY270810	*Shewanella schlegeliana* HRKA1 (NR_024792.1; 99%)	*Shewanella schlegeliana*
M27 and M28	Hi25	KY270805	*Shewanella schlegeliana* HRKA1 (NR_024792.1; 98%)	*Shewanella schlegeliana*
M30	Hi50	KY270807	*Shewanella schlegeliana* HRKA1 (NR_024792.1; 93%)	Unclassified Shewanellaceae

When several band numbers corresponding to the same accession number are listed, bands were individually excised and sequenced, showing however the same nucleotide sequence.

Table 6. Bands excised and identified (% similarity to nearest neighbour) from DGGE gels of digesta-associated intestinal bacteria (DAB). Numbers in the three right-hand columns show the number of samples (from six) per dietary group where a band at the same height of the excised one was found.

DGGE-band code	Isolation source	Accession no.	Nearest BLASTN match (GenBank accession no.; % sequence similarity)	Taxonomic classification
D4	H25	KY270786	*Carnobacterium divergens* NBRC 15683 (NR_113798.1; 100%)	*Carnobacterium divergens*
D6	H50	KY270787	*Carnobacterium divergens* NBRC 15683 (NR_113798.1; 99%)	*Carnobacterium divergens*
D9	H50	KY270789	*Carnobacterium divergens* NBRC 15683 (NR_113798.1; 99%)	*Carnobacterium divergens*
D16	H25	KY270792	*Staphylococcus pasteuri* ATCC 51129 (NR_114435.1; 99%)	*Staphylococcus pasteuri*
D17, D18 and D19	Hio, Hi25 and Hi50	KY270793	*Aeromonas rivipollensis* P2G1 (NR_144574.1; 100%)	*Aeromonas rivipollensis*
D14	H50	KY270790	*Kluyvera intermedia* JCM1238 (NR_112007.1; 99%)	*Kluyvera intermedia*
D3	H25	KY270785	*Citrobacter gillenii* CDC 4693-86 (NR_041697.1; 99%)	*Citrobacter gillenii*
D1, D2 and D12	Ho, H25 and H50	KY270784	*Shewanella schlegeliana* HRKA1 (NR_024792.1; 99%)	*Shewanella schlegeliana*
D15 and D20b	H50 and Ho	KY270791	*Shewanella schlegeliana* HRKA1 (NR_024792.1; 98%)	*Shewanella schlegeliana*

When several band numbers corresponding to the same accession number are listed, bands were individually excised and sequenced, showing however the same nucleotide sequence.

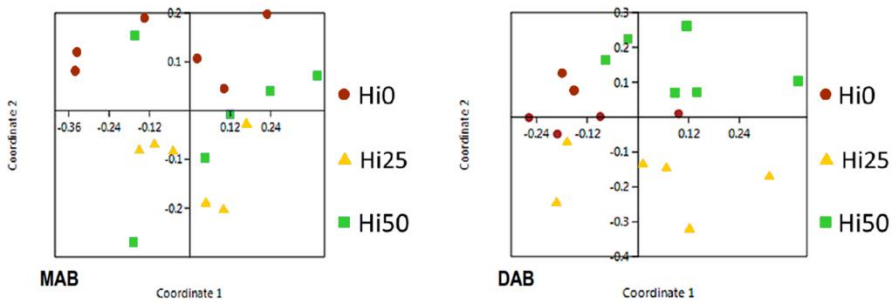

Fig 1. nMDS plots of mucosa- (left, MAB) and digesta-associated bacterial community (right, DAB) constructed on the band intensity matrix of the DGGE pattern using the Bray-Curtis index.

Paper 2

Differential response of mucosa and digesta associated gut microbiota to diet changes in seawater-phase Atlantic salmon (*Salmo salar*)

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Introduction

Atlantic salmon (*Salmo salar*) is the first most produced seawater species and one of the most economically important aquacultured organism worldwide (FAO, 2018). Its genome is sequenced (Lien et al., 2016) and the physiology of its organs is addressed in numerous studies. Still, there is one "organ" whose mechanisms and functions are not well understood. As a matter of fact, microbiota is considered a virtual organ for its adaptable and renewable metabolic activity (O'Hara and Shanahan, 2006). For instance, the number of bacteria found in human intestine is ten times higher than the host's entire body cells, contributing with around 540 000 genes (Evans et al., 2013). Similarly, each and every surface of the fish in contact with the external environment is covered by microorganisms, that are intertwined with a multitude of host endogenous functions; therefore, the host mucosa needs to sort microbes into pathogens and commensals (Pérez et al., 2010).

The mechanisms of the dialogue between host and guests are largely obscure. Some of the functions performed by the gut microbiota range from helping digestive and absorption processes (Dehler et al., 2017; Ray et al., 2012) to modulating lipid metabolism and energy balance (Falcinelli et al., 2015; Semova et al., 2012), from

dialoguing with the central nervous system through the so-called microbiota-gut-brain axis (Lyte and Cryan, 2014) to impacting aging (Smith et al., 2017) and social behaviour (Archie and Tung, 2015). The thorough taxonomic description of the gut microbiota composition is an essential step to gain mechanistic insights into the microbiota's role in fish metabolism and into the laws leading its establishment and upkeep. Ultimately, a milestone in the aquaculture production sector would be to know how to manipulate the microbiota to improve the welfare of the host (Peterson et al., 2009; Roeselers et al., 2011) and, eventually, to expand aquaculture production to feed the world with the highest environmental sustainability.

During the last decade, the microbiota living inside and on fish has been the object of valuable studies, with the main output being the description of the actors present or their putative potential functions (Boutin et al., 2014; Egerton et al., 2018; Llewellyn et al., 2014; Pérez et al., 2010; Tarnecki et al., 2017). Commonly, fish gut microbiota seems to be made up by Proteobacteria (62.5%), Firmicutes (15.2%), Bacteroidetes (6.0%), Actinobacteria (3.7%), Fusobacteria (2.9%), Planctomycetes (2.7%), Tenericutes (1.9%) and several other OTUs (Sullam et al., 2012). However, different results among the studies (due to salinity, trophic level, habitat, host phylogeny and many other factors (Sullam et al., 2012)) hinder the comprehension of fish microbiota ecology, function and role. Considering salmon gut, what is clear is that microbiota largely changes depending on intestinal tracts, on autochthonous (mucosa-adherent) vs allochthonous (transient with digesta) bacteria (Gajardo et al., 2016), and on diet (Bakke-McKellep et al., 2007; Hartviksen et al., 2014).

The research on feed used in aquaculture, a steeply rising food production sector (FAO, 2018), has worries about the ecological footprint of ingredients, specifically about the supply of protein ingredients. Nowadays, plant protein meals and fishmeal are the primary protein sources in aquafeed, but they raise questions about their economic, environmental and social sustainability (FAO, 2018). Protein from insects embodies an innovative possibility. In some cases, the use of insects instead of soymeal would lead to a lessened global warming potential in terms of N fertiliser savings (Salomone et al., 2017). Moreover, it is now ascertained that insect production has a lower environmental footprint than other land-based animals (van Huis et al., 2013) and it could contribute to economic, environmental and social sustainability (Halloran et al., 2018).

Changing the diet of fish has effects on gut microbiota, as well as on diet digestibility, final product quality and fish health and welfare. To the present, some studies addressed the modulation of rainbow

trout (*Oncorhynchus mykiss*) gut microbiota established by dietary *Hermetia illucens* larvae (H) meal. The studies generally agree on the fact that dietary insect increase gut microbiota diversity and probably lactic acid bacteria (Bruni et al., 2018; Huyben et al., 2019; Terova et al., 2019). Unfortunately, there was no general consensus on which tract and which type of sample to prioritise.

To the authors' knowledge, the literature does not report on previous study on the microbiota of Salmon fed with insects. To fill this gap, the present study aimed to describe the gut microbiota of seawater Salmon fed with H meal with high-throughput sequencing of the V1-V2 16S rRNA gene.

Materials and methods

Experimental fish, diet and sample collection

A. Salmon were reared for 114 days in 12 sea-cages (5×5×5 m; 125 m^3; 90 fish per cage, n=3 replicates) at the Gildeskål Research Station (GIFAS) in Langholmen, Inndyr, Norway (67°N, Northern Norway), in accordance with the ethical standards approved by the European Directive 2010/63/EU on the protection of animals used for scientific purposes. Fish were fed by hand until visual satiation one of four isoproteic, isoenergetic and isolipidic diets: a control diet (insect meal, H0, 0% of H meal inclusion) and three diets with increasing substitution levels of the fishmeal with H meal (33, 66 or 100%; H33, H66 and H100, respectively). The insect larvae were grown on media partially containing organic waste streams mixed with the macroalga *Ascophyllum nodosum* (40:60) to increase the level of long chain polyunsaturated fatty acids of the insects. Further details on the diets and the rearing trial are available in Belghit et al. (2019).

When fish reached the commercial weight, they were randomly taken form the tanks and sacrificed by a sharp blow to the head.

After decontaminating the exterior surface of the abdomen with 70% ethanol, the distal intestine was dissected aseptically and opened longitudinally. Only fish with chyme along the whole intestine were sampled to ensure that the intestine had been exposed to the diets until the point of sampling. The intestinal content was collected into a 1.5 ml sterile tube using a spatula. The gut tissue was rinsed in sterile PBS three times to get rid of traces of digesta and cut into 3 pieces: one for mucosa-associated bacteria profiling and the others for gene expression and histological evaluation. The collection of microbiota samples was performed near a flame. All tools were sprayed with 70% ethanol and burnt in between samples. The

intestinal content and tissue were snap frozen in liquid N_2 and stored at -80 °C before further processing.

DNA extraction

Total DNA was extracted from 200 mg distal-gut digesta or mucosa using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany; catalog no., 51504) with some modifications to the manufacturer's specification as described by Gajardo et al. (2016), except that 2 mL prefilled bead tubes (Qiagen; catalog no., 13118-50) were used for the bead beating. For quality control purposes, a companion "blank extraction" sample was added to each batch of sample DNA extraction by omitting the input material, whereas an additional ZymoBIOMICS™ microbial community standard (Zymo Research, California, USA; catalog no., D6300) was included for each DNA extraction kit as a positive control.

Amplicon PCR

The V1-2 hypervariable regions of bacterial 16S rRNA gene were amplified using the primer set 27F (AGA GTT TGA TCM TGG CTC AG) and 338R (GCW GCC WCC CGT AGG WGT) (Roeselers et al., 2011). The PCR was run in a total reaction volume of 25 µL containing 12.5 µL of Phusion® High-Fidelity PCR Master Mix (Thermo Scientific, CA, USA; catalog no., F531L), 10.9 µL molecular grade H_2O, 1 µL DNA template and 0.3 µL of each primer (10 µM). The amplification program was set as follows: initial denaturation at 98 °C for 3 min; 35 cycles of denaturation at 98 °C for 15 s, annealing decreasing from 63 °C to 53 °C in 10 cycles for 30 s followed by 25 cycles at 53 °C for 30 s, and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 10 min. For samples with faint or invisible bands in the agarose gel after PCR, the PCR condition was optimized by applying serial dilutions to the DNA templates to reduce the influence of PCR inhibitors. All the digesta samples were diluted 1:2 in buffer ATE (10 mM Tris-Cl pH 8.3 with 0.1 mM EDTA and 0.04% NaN_3) whereas all the mucosa samples were diluted 1:32. The formal amplicon PCR was run in duplicate incorporating two negative PCR controls, which were generated by replacing the template DNA with molecular grade H_2O. The duplicate PCR products were then pooled and examined by a 1.5% agarose gel electrophoresis.

Quantification of 16S rRNA gene by qPCR

To assist identifying contaminant sequences, the 16S rRNA gene quantity in the diluted DNA templates used for the amplicon PCR was measured by qPCR. The qPCR assays were performed using a universal primer set (forward, CCA TGA AGT CGG AAT CGC TAG; reverse, GCT TGA CGG GCG GTG T) that has been used for bacterial

DNA quantification in previous studies (Vandeputte et al., 2017, Ramseier et al., 2009). The assays were carried out using the LightCycler 96 (Roche Applied Science, Basel, Switzerland) in a 10 µL reaction volume, which contained 2 µL of PCR-grade water, 1 µL diluted DNA template, 5 µL LightCycler 480 SYBR Green I Master Mix (Roche Applied Science) and 1 µL (3 µM) of each primer. Samples, together with the extraction blanks and mock, were run in duplicate in addition to Femto™ bacterial DNA standards (Zymo Research; catalog no., E2006) and a no-template control of the qPCR assay. The qPCR program encompassed an initial enzyme activation step at 95 °C for 2 min, 45 three-step cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 15 s, and a melting curve analysis at the end. Quantification cycle (Cq) values were determined using the 'Second Derivative Maximum Method' (Rasmussen, 2001). The specificity of qPCR amplification was confirmed by evaluating the melting curve of qPCR products and the band pattern on the agarose gel after electrophoresis. The inter-plate calibration factor was calculated following the method of Hellemans et al. (2007), using the bacterial DNA standards as inter-plate calibrators.

Sequencing

The sequencing was carried out on a Miseq platform following the Illumina 16S metagenomic sequencing library preparation protocol (Illumina, 2013). Briefly, the PCR products were cleaned using the Agencourt AMPure XP system (Beckman Coulter, Indiana, USA; catalog no., A63881), multiplexed by dual indexing using the Nextera XT Index Kit (Illumina, California, USA; catalog no., FC-131-1096) and purified again using the AMPure beads. After the second clean-up, representative libraries were selected and analyzed using the Agilent DNA 1000 Kit (Agilent technologies, California, USA; catalog no., 5067-1505) to verify the library size. Cleaned libraries were quantified using the Invitrogen Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, California, USA; catalog no., Q32854), diluted to 4 nM in 10 mM Tris (pH 8.5) and pooled in an equal volume. Negative controls with library concentrations lower than 4 nM were pooled in the equal volume directly. Due to the low diversity of amplicon library, 15% Illumina generated PhiX control (catalog no., FC-110-3001) was spiked in by combining 510 µL amplicon library with 90 µL PhiX control library. The library was loaded at 6 pM and sequenced using the Miseq Reagent Kit v3 (600-cycle) (Illumina; catalog no., MS-102-3003).

Sequence data processing

The raw sequence data were processed by the DADA2 pipeline to infer amplicon sequence variants (ASVs) (Callahan et al., 2016). Specifically, the demultiplexed paired-ended reads were trimmed off

the primer sequences (forward reads, first 20 bps; reverse reads, first 18 bps) and truncated at the position where the median of Phred quality score dropped dramatically (forward reads, at position 290 bp; reverse reads, at position 248 bp). After trimming and filtering, the run-specific error rates were estimated and the ASVs were inferred by pooling reads from all the samples sequenced in the same run. The chimeras were removed using the "pooled" method after merging the reads. The resulting ASV table and representative sequences were imported into QIIME2 (version, 2019.7) (Bolyen et al., 2019). The taxonomy was assigned by a scikit-learn naive Bayes machine-learning classifier (Bokulich et al., 2018), which was trained on the SILVA 132 99% OTUs (Quast et al., 2012) that were trimmed to only include the regions of 16S rRNA gene amplified by our primers. Taxa identified as chloroplast or mitochondria were excluded from the ASV table. The feature table was conservatively filtered to remove ASVs that had no phylum-level taxonomic assignment or appeared in only one sample. Contaminant sequences were removed from the ASV table based on the criteria suggested by Davis et al. (2018), i.e., contaminants are often found in negative controls and inversely correlate with sample DNA concentration. After removing contaminant sequences, the taxonomic composition of the mock was evaluated by the q2-quality-control plugin. The ASVs filtered from the raw ASV table were also removed from the representative sequences, which were then aligned by the MAFFT (Katoh and Standley, 2013). A phylogenetic tree was built from the masked alignment using the FastTree (Price et al., 2010). The core microbiota and alpha-diversity indices were computed using the ASV table collapsed at the species level. The core microbiota was calculated based on the 80% prevalence threshold and visualized by the Venn's diagram. The alpha-diversity indices, including Observed species, Pielou's Evenness, Shannon's index and Faith's phylogenetic diversity (PD), were computed via the R package *microbiome* (Lahti et al., 2017) and *picante* (Kembel et al., 2010). For beta-diversity analyses, we used distance matrices including Jaccard distance, unweighted-UniFrac distance, Aitchison distance and PHILR transformed Euclidean distance. Since rarefying remains to be the best solution for unweighted distance matrices (Weiss et al., 2017), the Jaccard distance and unweighted-UniFrac distance were calculated using a rarefied ASV table subsampled with 2047 sequences per sample. The compositional data aware distance matrices, Aitchison distance and PHILR transformed Euclidean distance, were calculated using the unrarefied ASV table. The Aitchison distance was computed by the *DEICODE* plugin in QIIME2, a form of Aitchison distance that is robust to high levels of sparsity by using the matrix completion to handle the excessive zeros in the microbiome data (Martino et al., 2019). The

phylogenetic isometric log-ratio transform of the ASV table was performed in R using the *philr* package (Silverman et al., 2017). The distance matrices were visualized by the principal coordinates analysis (PCoA).

Association analysis

The ASV table was collapsed at the genus level before running the multivariate association analysis. Bacterial taxa of very low abundance (< 0.01%) or low prevalence (present in < 25% of samples) were removed from the feature table. The microbial clades were then tested for significant associations with metadata of interest by MaAsLin2 (version, 0.99.12) (https://huttenhower.sph.harvard.edu/maaslin2) in R, using the default program parameters. The results of the analysis are the associations of specific microbial clades with metadata, deconfounding the influence of other factors included in the model. Association was considered significant when the *q*-value was below 0.25. Metadata included in the multivariate association testing are fixed factors Diet + Sample origin + distal intestine somatic index (DISI) + lamina propria cellularity (histological scores) + immune response (qPCR) + barrier function (qPCR), and random factors FishID + NetPen. FishID was nested in NetPen, and NetPen nested in Diet. Lamina propria cellularity reflects the severity of inflammation in the distal intestine. Based on the degree of cellular infiltration within the lamina propria, a value of normal, mild, moderate, marked or severe was assigned. To make the data appropriate for the association testing, the highly skewed five-category scores were collapsed into more balanced binary data, i.e., normal and abnormal. The immune genes included for the association testing were myeloid differentiation factor 88 (*myd88*), interleukin 1β (*il1β*), interleukin 8 (*il8*), cluster of differentiation 3 γδ (*cd3γδ*), transforming growth factor β1 (*tgfβ1*), interferon γ (*ifnγ*), interleukin 17A (*il17a*), fork-head box P3 (*foxp3*) and interleukin 10 (*il10*), whose expression levels were higher in the distal intestine of fish assigned abnormal regarding lamina propria cellularity. Since the expression levels of immune genes were highly correlated, we ran a principle component analysis (PCA) and extracted the first principle component for the association testing to avoid multicollinearity and reduce the number of association tests. For genes relevant to the barrier function, which included claudin-15 (*cldn15*), claudin-25b (*cldn25b*), zonula occludens 1 (*zo1*), E-cadherin / cadherin 1 (*cdh1*) and mucin-2 (*muc2*), we also used the first principle component of the PCA for the association testing based on the same considerations. The R package *PerformanceAnalytics* (Peterson and Carl, 2019) and *factoextra*

(Kassambara and Mundt, 2017) was used for exploring the correlations and principle component analysis, respectively.

Statistics

All the statistical analyses were run in R except for the PERMANOVA, which was run in PRIMER v7. The differences in the alpha-diversity indices were compared using linear mixed effects models via the *lme4* package (Bates et al., 2015). Predictor variables in the models included the fixed effects Diet + Sample origin + Diet x Sample origin, and the random effects FishID + NetPen. The models were validated by visual inspections of residual diagnostic plots generated by the *ggResidpanel* package (Goode and Rey, 2019). The statistical significance of fixed predictors was estimated by Type III *ANOVA* with Kenward-Roger's approximation of denominator degrees of freedom via the *lmerTest* package (Kuznetsova et al., 2017). When the interaction between the main effects was significant, conditional contrasts for the main effects were made via the *emmeans* package (Lenth, 2019). To compare the differences in beta-diversity, we performed the PERMANOVA (Anderson, 2001) using the same predictors included in the linear mixed effects models. Terms with negative estimates for components of variation were sequentially removed from the model via term pooling, starting with the one showing the smallest mean squares. At each step, the model was reassessed whether more terms need to be removed or not. Conditional contrasts for the main effects were constructed when their interaction was significant. Monte Carlo *p* values were computed as well when the unique permutations for the terms in the PERMANOVA were small (< 100). The homogeneity of multivariate dispersions among groups was visually assessed with boxplots and was formally tested by the permutation test, PERMDISP (Anderson, 2006), via the R package *vegan* (Oksanen et al., 2019). Multiple comparisons were adjusted by the Benjamini-Hochberg procedure where applicable. Differences were regarded as significant when $p < 0.05$.

Availability of data and code

Raw sequence data and sample metadata are available from the NCBI SRA database (https://www.ncbi.nlm.nih.gov/sra) with BioProject PRJNA555355. Codes used for sequence processing and data analysis will be deposited at a Github repository and will be archived at Zenodo (https://zenodo.org/).

Results and Discussion

Summary and highlights of the main findings

This study compared the distal gut microbiota of A. salmon fed a commercially relevant diet or an insect meal-based diet. To the authors' knowledge, the literature provides no studies on the effect of dietary insect on the gut microbiota of A. salmon. This study also investigated the dissimilarities between digesta- and mucosa-associated microbiota and identified associations between microbial clades and host responses. Microbiota composition, biodiversity and differential abundance were affected by both diet and sample-origin (Figure 1), as previously portrayed (Sullam et al., 2012). The core microbiota of all the samples comprised *Aliivibrio*, *Brevinema andersonii*, and *Mycoplasma* (Figure 2). IM samples showed higher alpha-diversity indices in comparison to REF samples (Figure 3), seemingly a typical trait occurring when feeding the salmonid rainbow trout (*Oncorhynchus mykiss*) with insects (Bruni et al., 2018; Huyben et al., 2019). Furthermore, as firstly revealed by Gajardo and colleagues (2017), the present study highlighted strong differences in composition and biodiversity between content and mucosa samples, and a lower presence of taxa in the mucosa samples. Besides, diet seemed to exert a larger effect on digesta sample than on the mucosa samples. Beta-diversity data produced with PCoA (Figure 4) and PERMANOVA (Table 1) agreed by saying that, regardless of the distance matrix used, both diet and sample origin factors had a role in shaping different beta-diversities. Finally, results of the multivariate association analysis highlighted that 53 taxa had significant associations with the metadata of interest (Figure 5).

Core microbiota

In total, 108 taxa were identified as core microbiota based on their prevalence in each sample type (Figure 2; Table S2). Specifically, *Aliivibrio*, *Brevinema andersonii*, and *Mycoplasma* were identified as core microbiota in all the sample types. Additionally, ten taxa were identified as core microbiota of digesta-associated samples (REF-DIC and IM-DIC), which included *Bacillus*, *Corynebacterium 1*, *Lactobacillus* (*L. aviaries, L. fermentum* and two unclassified species), *Leuconostoc*, *Parageobacillus toebii*, *Ureibacillus* and *Weissella*. RsaHF231, *Actinomyces*, *Corynebacterium aurimucosum* ATCC 70097 and *Microbacterium* were unique to the fish fed the IM diet, shared between the mucosa and the digesta samples. No additional core microbiota was identified for the mucosa-associated samples (REF-DIM and IM-DIM). Notably, 86 taxa were found to be more prevalent in IM-DIC than in other sample types.

Commonly, fish gut microbiota seems to be made up by Proteobacteria (62.5%), Firmicutes (15.2%), Bacteroidetes (6.0%), Actinobacteria (3.7%), Fusobacteria (2.9%), Planctomycetes (2.7%), Tenericutes (1.9%) and several other OTUs (Sullam et al., 2012). In the present study, the results showed that fish gut were mainly populated by Proteobacteria, Firmicutes, Tenericutes, Spirochaetes, Actinobacteria and RsaHF231; Proteobacteria, Firmicutes and Tenericutes were present in all the four different sample types. These results agree with other studies analyzing the gut microbiota of A. salmon fed diets containing ingredients of commercial interest (Dehler et al., 2017; Gajardo et al., 2017; Hartviksen et al., 2014). In our study, the core microbiota was composed of *Aliivibrio*, *Brevinema andersonii* and *Mycoplasma*. *Aliivibrio* spp. characterized A. salmon reared in a commercial mariculture system during austral summer at above 16 °C, while its abundance decreased after summer (Zarkasi et al., 2014). Bacteria belonging to the family Brevinemataceae were previously found in A. salmon distal intestine mucosa (Gajardo et al., 2016) but also in rainbow trout distal gut fed either control or treatment diet (Lyons et al., 2016) and in grass carp, crucian carp and bighead carp cohabiting in the same pond (Li et al., 2015). Finally, it was suggested that *Mycoplasma* be a natural member of A. salmon microflora (Holben et al., 2002). In fact, some studies found this genus abundantly in all fish, other research found it at high levels in few of the examined fish and some other studies found the genus at much lower levels than other dominant microbial groups (Hartviksen et al., 2014; Llewellyn et al., 2016; Zarkasi et al., 2014). *Mycoplasma* have "unusual nutritional requirement for sterols and other serum factors", having a fermentative metabolism they mainly produce lactic acid and acetic acid, and were thought to be saprophytes (Holben et al., 2002; Zarkasi et al., 2014).

Bacteria belonging to the Spirochaetes phylum were also found in all the groups of the present study, although their abundance showed very large interindividual variability and only two samples of the IM-DIC group contained bacteria belonging to this phylum. Spirochaetes were previously retrieved in several studies on salmonid gut microbiota fed commercial and experimental diets (Betiku et al., 2018; Ingerslev et al., 2014; Jaramillo-Torres et al., 2019; Lyons et al., 2017). Less than 5% of the total bacterial abundance was taken by RsaHF231 in all IM-DIC samples and in around the half of the IM-DIM samples; RsaHF231 have not been documented in fish before, but it were recently found in *H. illucens* gut (Jiang et al., 2019). Few Fusobacteria were recovered in REF-DIC only. This result aligns with the main works on A. salmon in the literature, where Fusobacteria have been retrieved in the intestinal contents of A. salmon (Dehler et al., 2017a;

Gajardo et al., 2017, 2016; Zarkasi et al., 2016). Finally, a meager number of Bacteroidetes was found in the majority of the IM-DIC and in a few of the samples belonging to the other three groups. Typically, Bacteroidetes are found in both freshwater and seawater A. salmon digesta fed commercial-like diets (Dehler et al., 2017b, 2017a; Zarkasi et al., 2016) and they were also recovered from mucosa-associated samples of A. salmon fed commercial-like diets containing either fishmeal or plant-derived proteins or poultry meal by Gajardo et al. (2017, 2016). Bacteroidetes were also found in rainbow trout mid gut mucosa fed either a fishmeal-based diet or a diet containing full-fat *Tenebrio molitor* meal (Antonopoulou et al., 2019), while in rainbow trout digesta and mucosa fed H meal Bacteroidetes accounted for a small quantity (Bruni et al., 2018; Huyben et al., 2019).

Alpha- and beta-diversity

All the alpha-diversity indices showed similar results with regard to the main effects, diet and sample origin. The IM diet resulted in higher alpha-diversity indices than those of the REF diet ($p < 0.05$), whereas digesta-associated samples showed higher alpha-diversity indices than those of mucosa-associated samples ($p < 0.05$) (Figure 4). A significant interaction between the main effects was noted in the observed species ($p = 0.024$). The difference in the observed species between the diets was more profound in digesta-associated samples than that in mucosa-associated samples.

The PCoA results are shown in Figure 4. The two upper plots (Figure 4A-B), built using the Jaccard and unweighted UniFrac distance matrix respectively, showed clear separations of samples belonging to different diets and sample origins. Notably, the average distance between samples from different dietary groups was dependent on sample origin, which was shorter in mucosa-associated samples than that in digesta-associated samples. The two lower plots (Figure 4C-D), built on the Aitchison and PHILR transformed Euclidean distance matrix respectively, also showed separations of samples belonging to different diets and sample origins. Again, the average distance between samples from different dietary groups was dependent on sample origin. Specifically, mucosa-associated samples from different dietary groups formed clusters boarding (Figure 4C) or overlapping (Figure 4D) each other, whereas digesta-associated samples from different dietary groups were clearly separated.

The PERMANOVA and its following conditional contrasts confirmed the PCoA results. Regardless of the distance matrix used, both main factors had significant effects on the beta-diversity and their interaction was significant as well ($p < 0.05$) (Table 1). The conditional contrasts revealed that the diet effect on the beta-

diversity, when measured by the PHILR transformed Euclidean distance, was mostly due to differences in the digesta-associated samples rather than mucosa-associated samples (REF-DIC VS. IM-DIC, p = 0.001; REF-DIM VS. IM-DIM, p = 0.069).

The results on the homogeneity of multivariate dispersions are shown in Table 2. For Jaccard distance, significant differences in the multivariate dispersions were observed for samples of different origins regardless of the diets (REF-DIC VS. REF-DIM, p = 0.043; IM-DIC VS. IM-DIM, p = 0.002), in addition to digesta-associated samples from different dietary groups (REF-DIC VS. IM-DIC, p = 0.002). For unweighted UniFrac distance, REF-DIC showed higher multivariate dispersions than other sample types resulting in significant differences compared to REF-DIM (p = 0.002) and IM-DIC (p = 0.002). For Aitchison distance, REF-DIM showed lower multivariate dispersions than other sample types resulting in significant differences compared to REF-DIC (p = 0.042) and IM-DIM (p = 0.042). For PHILR transformed Euclidean distance, the differences in the multivariate dispersions among the sample types were not significant (p > 0.05).

Diet effect

It is commonly accepted that changing the diet of fish has effects on gut microbiota and our investigations align with that, as documented in Figures 1-5. To the present, some studies addressed the modulation of rainbow trout gut microbiota established by dietary H meal. The present results are globally in line with studies on gut microbiota of rainbow trout fed diets where the conventional protein sources had been substituted by either H meal or *Tenebrio molitor* meal (Antonopoulou et al., 2019; Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019). Specifically, the insect increased microbiota diversity and probably lactic acid bacteria when rainbow trout was fed H meal (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019), while Antonopoulou et al. (2019) noted that *T. molitor* decreased the number of OTUs. Another study enquired the presence of *Enterobacteriaceae*, *Clostridium leptum*, *C. coccoides*, *Lactobacillus sp.* and *Enterococcus sp.* using fluorescent *in situ* hybridisation in the intestine content of rainbow trout fed diets containing either *T. molitor*, *Gryllodes sigillatus*, *Blatta lateralis* or H meals was (Józefiak et al., 2019); the authors found an increased number of bacteria in the insect-fed groups. Unfortunately, despite the mentioned works align by suggesting that dietary insects increase overall richness and lactic acid bacteria, it is difficult to unambiguously compare the studies, as the trials used different

experimental factors, such as insect origin, sample type collected, fish size, rearing duration, etc.

Sample origin effect

The taxonomic composition of the mucosa-associated samples showed higher similarity than that of the digesta-associated samples, which were more diet-dependent (Figure 1). At phylum level, the dominant taxa of mucosa-associated samples were Spirochaetes, Proteobacteria, Firmicutes and Tenericutes. For digesta-associated samples, the dominant taxa of REF-DIC were Tenericutes, Proteobacteria, Firmicutes and Spirochaetes, whereas IM-DIC was dominated by Firmicutes, Actinobacteria, Proteobacteria, Tenericutes and RsaHF231 (Figure 1A). At genus level, the dominant taxa of mucosa-associated samples were *Brevinema*, an unclassified taxon of Spirochaetaceae family, *Aliivibrio* and *Mycoplasma*. For digesta-associated samples, the dominant taxa of REF-DIC were *Mycoplasma*, *Aliivibrio*, *Photobacterium*, *Brevinema and Lactobacillus*, whereas IM-DIC was dominated by *Aliivibrio*, an unclassified taxon of Lactobacillales order, *Corynebacterium 1*, *Bacillus*, *Mycoplasma* and *Actinomyces* (Figure 1B).

Although the effect of sample origin has been investigated by a modest number of papers, the literature reports that mucosa-associated bacterial community is less abundant than digesta-associated bacterial community (Gajardo et al., 2017; Hartviksen et al., 2014). Also the present study highlighted a low relative abundance in mucosa samples in comparison to digesta samples; additionally, mucosa samples only contained OTUs already present in the digesta samples. These findings suggest that bacteria transient with the intestinal content undergo a specific selection that allows a small number to adhere to the mucosa.

It seems interesting to note how digesta samples, irrespective of dietary group, shared six bacteria attributable to the Lactobacillales order and three bacteria attributable to the Bacillales order, in addition to several other Firmicutes at less than 80% prevalence. On the other hand, in the mucosa-associated communities, no bacteria of the Firmicutes phylum was shared between the samples, except for a very few bacteria of the Firmicutes phylum (mainly Enterococcus, Oceanobacillus and Bacillus) shared in less than 80% of the samples. The role of lactic acid bacteria in fish species is debated.

Significant associations between microbial clades and gene expression data

The multivariate association analysis identified 53 taxa showing significant associations with the metadata of interest (Figure 5A). In total, 37 differentially abundant taxa were identified for the diet effect, 27 of which showed increased relative abundances in salmon fed the IM diet as partially illustrated in Figure 5B. Forty-eight differentially abundant taxa were identified for the sample origin effect, 46 of which, including *Bacillus*, *Enterococcus*, *Flavobacterium*, *Lactobacillus*, *Leuconostoc*, *Mycoplasma*, *Peptostreptococcus*, *Photobacterium* and *Weissella*, showed lower relative abundances in the mucosa-associated samples. Notably, *Brevinema andersonii* and an unclassified taxon of the Spirochaetaceae family were enriched in the mucosa-associated samples (Figure 5C). For the histological scores, the relative abundance of an unclassified taxon of the Sphingobacteriaceae family and RsaHF231 phylum were found to increase and decrease, respectively, in fish scored abnormal regarding distal gut lamina propria cellularity (lpc). The relative abundance of *Acinetobacter* and *Pseudomonas* were negatively correlated with the distal intestine somatic index (DISI). Six taxa, including *Actinomyces*, *Brevinema andersonii*, the unclassified taxon of the Sphingobacteriaceae family, *Kurthia*, *Lysobacter* and *Microbacterium*, were found to associate with the expression of genes related to immune responses. Notably, the relative abundance of *Brevinema andersonii* showed a clear positive correlation with the expression levels of the immune genes (Figure 5D), which decrease as the first principle component of the PCA increases. Furthermore, 4 taxa including *Cellulosimicrobium*, the unclassified taxon of the Spirochaetaceae family, *Glutamicibacter* and *Pseudomonas* were found to associate with the expression of genes related to barrier functions. Notably, the relative abundance of the unclassified taxon of the Spirochaetaceae family also showed a negative correlation with the expression levels of the barrier function relevant genes (Figure 6E), which also decrease as the first principle component of the PCA increases.

Mock and negative controls

All the eight bacterial taxa included in the mock were successfully identified at the genus level, with *Enterococcus faecalis, Lactobacillus fermentum, Listeria monocytogenes* and *Staphylococcus aureus* further being annotated at the species level (Figure 6A). The average Pearson's correlation coefficient for the expected and observed taxa at the genus level was 0.33. The relative abundance of most Gram-positive bacteria, *L. monocytogenes* and *E. faecalis* in particular, was

underestimated. In contrast, the relative abundance of Gram-negative bacteria was slightly overestimated. As the observed mock composition is slightly different that the expected composition, generalizations on the observed composition of the samples should be avoided. Fortunately, the two mocks analyzed produced very similar results and suggested that the flow of the analyses is consistent from sample to sample. Most ASVs (97.6% - 99.9%) in the extraction and library blanks were classifed as *Pseudomonas* (Figure 6B), which were the main contaminant taxa removed from the samples besides *Hymenobacter*, Cutibacterium, *Brevundimonas*, *Curtobacterium*, *Modestobacter*, *Sphingomonas*, *Micrococcus* and other rare taxa. The presence of the mentioned contaminants confirms that lab tools and reagents contain contaminant sequences (Salter et al., 2014).

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Table 1. PERMANOVA results and conditional contrasts. The fixed effects Diet + Sample origin + Diet x Sample origin, and the random effects FishID + NetPen, were used built on different distance matrices.

Distance matrix	Main effects			Conditional contrasts			
	Diet	Origin	Interaction	Diet: REF *vs* IM		Origin: Contents *vs* Mucosa	
				Origin: Contents	Origin: Mucosa	Diet: REF	Diet: IM
Jaccard	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Unweighted UniFrac	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Aitchison	0.001	0.005	0.004	0.001	0.001	0.002[1]	0.001[1]
PHILR (Euclidean)	0.001	0.001	0.001	0.001	0.069	0.001	0.001

[1]*Monte Carlo P values.*

Table 2. Homogeneity of multivariate dispersions.

Distance matrix	Conditional contrasts			
	Diet: REF *vs* IM		Origin: Contents VS. Mucosa	
	Origin: Contents	Origin: Mucosa	Diet: REF	Diet: IM
Jaccard	0.001	0.076	0.032	0.001
Unweighted UniFrac	0.001	0.06	0.001	0.071
Aitchison	0.453	0.02	0.021	0.276
PHILR (Euclidean)	0.195	0.656	0.448	0.331

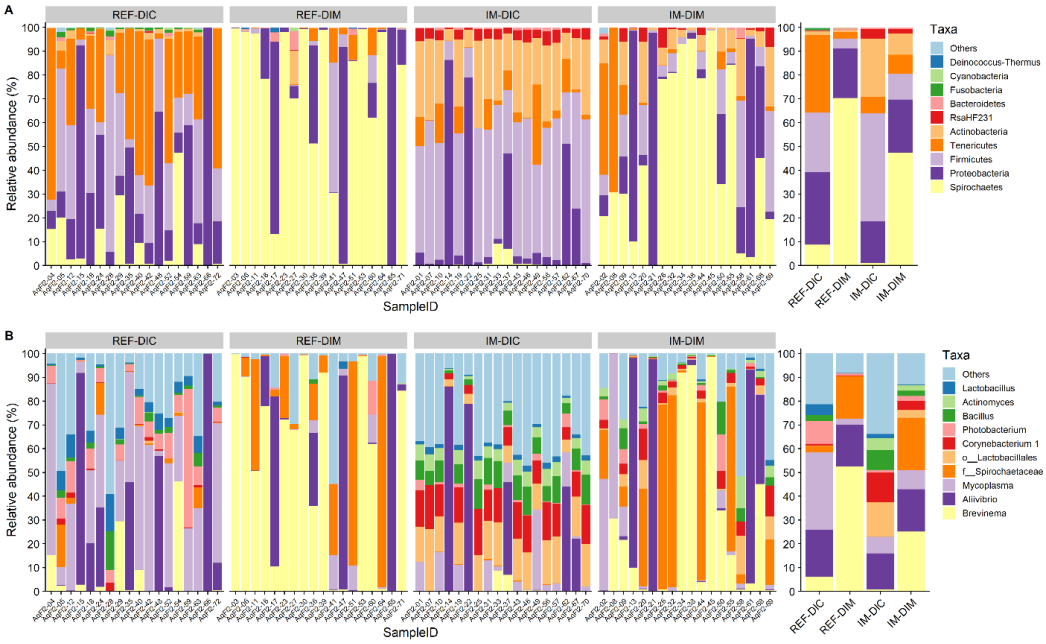

Fig. 1 Top 10 most abundant taxa of digesta- and mucosa-associated distal gut microbiota in Atlantic salmon fed different diets. The top 10 most abundant taxa were displayed at phylum (A) and genus (B) level, with their mean relative abundance within each sample type being plotted on the right side. o__, order; f__, family; REF, reference diet; IM, insect meal diet; DIC, distal intestine content; DIM, distal intestine mucosa.

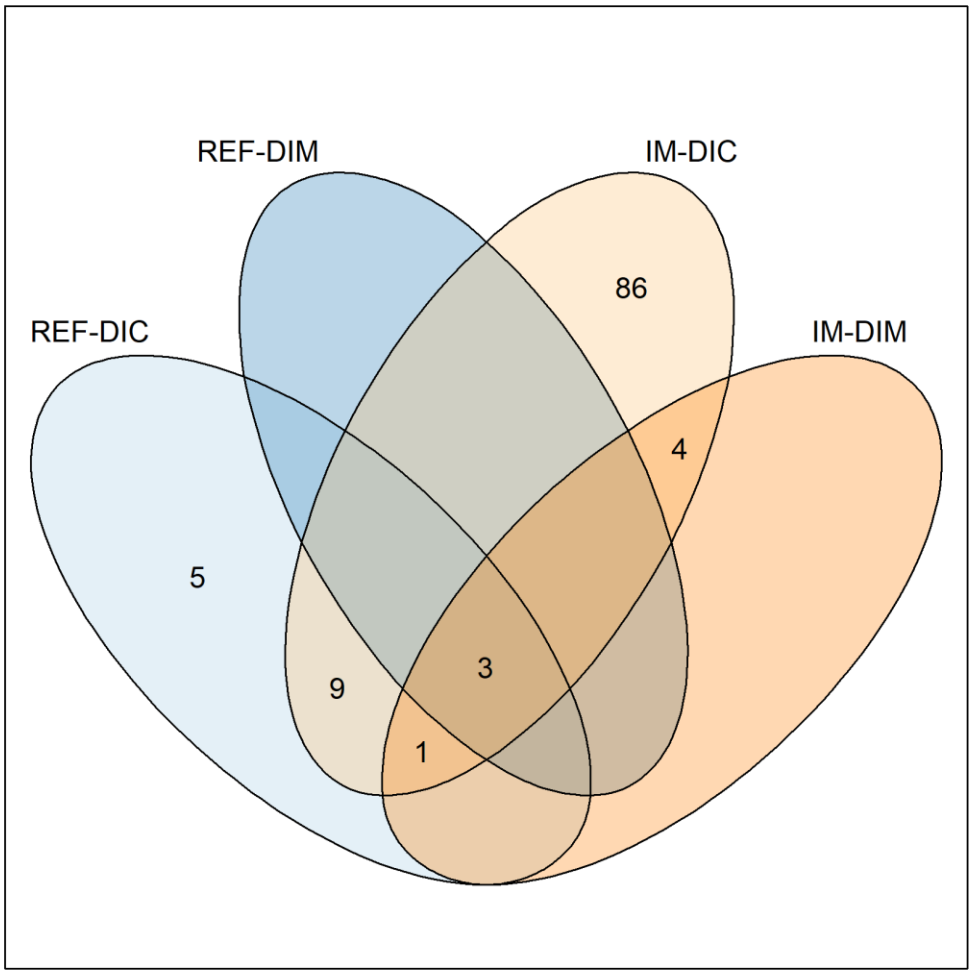

Fig. 2 The core taxa of digesta- and mucosa-associated distal gut microbiota in Atlantic salmon fed different diets. The core taxa were computed based on the 80% prevalence threshold for each sample type. REF, reference diet; IM, insect meal diet; DIC, distal intestine content; DIM, distal intestine mucosa.

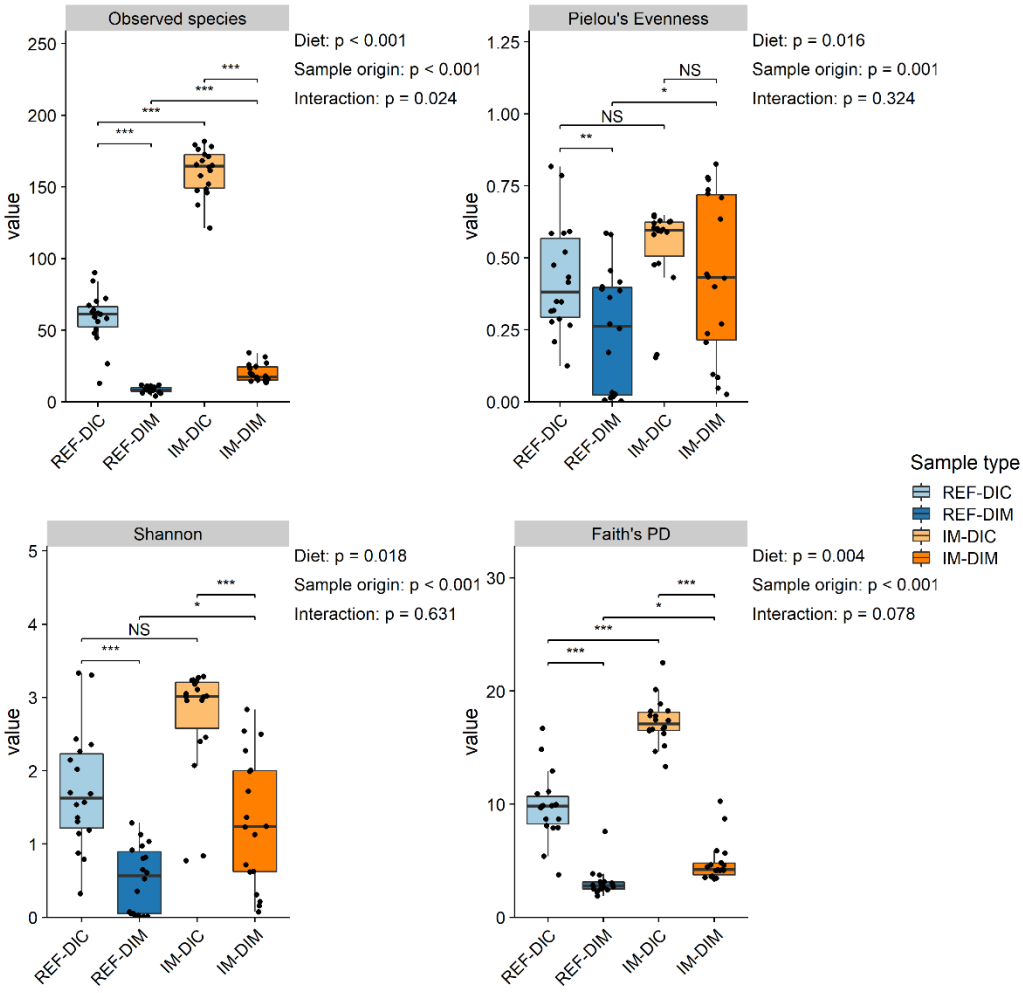

Fig. 3 Alpha-diversity of digesta- and mucosa-associated distal gut microbiota in Atlantic salmon fed different diets. The *p* value of the main effects and their interaction were displayed on the top-right corner of each sub-plot. Asterisks denote statistically significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). PD, phylogenetic diversity; REF, reference diet; IM, insect meal diet; DIC, distal intestine content; DIM, distal intestine mucosa.

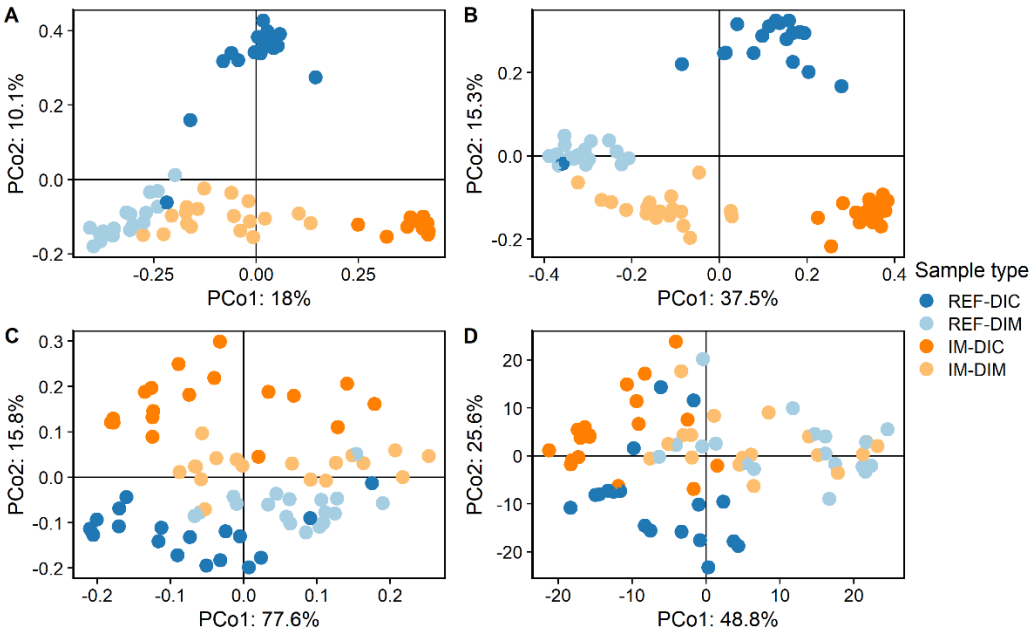

Fig. 4 Beta-diversity of digesta- and mucosa-associated distal gut microbiota in Atlantic salmon fed different diets. The PCoA plots were built on Jaccard (A), unweighted UniFrac (B), Aitchison (C) and PHILR transformed Euclidean (D) distance matrix, respectively. PCo, principle coordinate; PHILR, phylogenetic isometric log-ratio; REF, reference diet; IM, insect meal diet; DIC, distal intestine content; DIM, distal intestine mucosa.

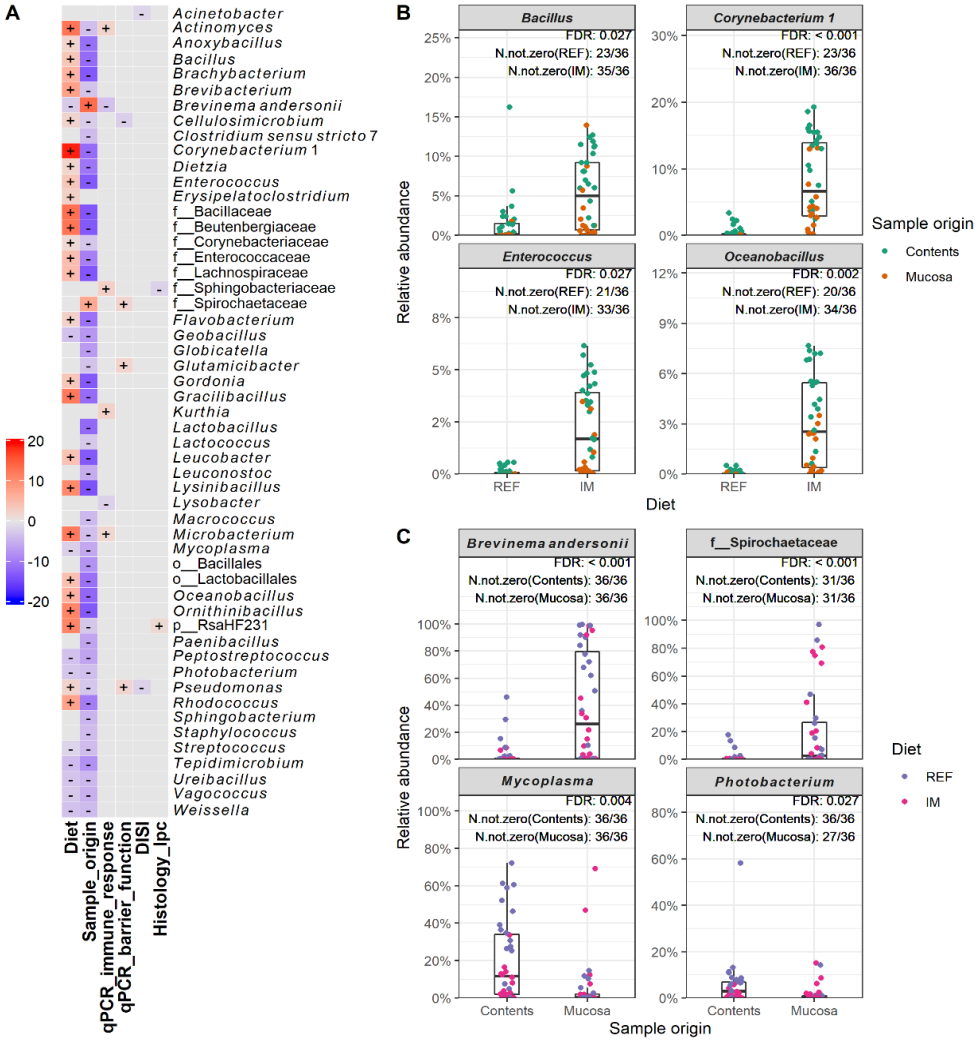

Fig. 5 Significant associations between distal gut microbiota and sample metadata in Atlantic salmon. (A) Heatmap summarizing all significant associations between microbial clades and sample metadata. Cells that denote significant associations are colored by *q*-value (-log(q-value) * sign(coefficient)) and overlaid with a plus (+) or minus (-) sign that indicates the direction of association: Diet (+), higher abundance in salmon fed the IM diet; Sample_origin (+), higher abundance in mucosa-associated samples; Histology_lpc (+), higher abundance in salmon scored abnormal regarding lamina propria cellularity (lpc) in the distal gut; DISI (+), positive correlation between microbial clade abundance and distal intestine somatic index (DISI); qPCR_immune_response (+) / qPCR_barrier_function (+), negative correlation between microbial clade abundance and the gene expression levels. (B) Representative taxa showing increased relative abundance in salmon fed the IM diet. (C) Taxa that were enriched in the distal gut mucosa of salmon. (D) Association between *Brevinema andersonii* and the immune genes. Note that expression levels of the immune genes decrease as the first principle component (PC1) of the PCA increases (Figure S2). (E) Association between an unclassified taxon of the Spirochaetaceae family and the barrier function relevant genes. Also note that expression levels of the barrier function relevant genes decrease as the PC1 increases (Figure S4). p__, phylum; o__, order; f__, family; FDR, false discovery rate; N.not.zero, number of observations that are not zero; REF, reference diet; IM, insect meal diet.

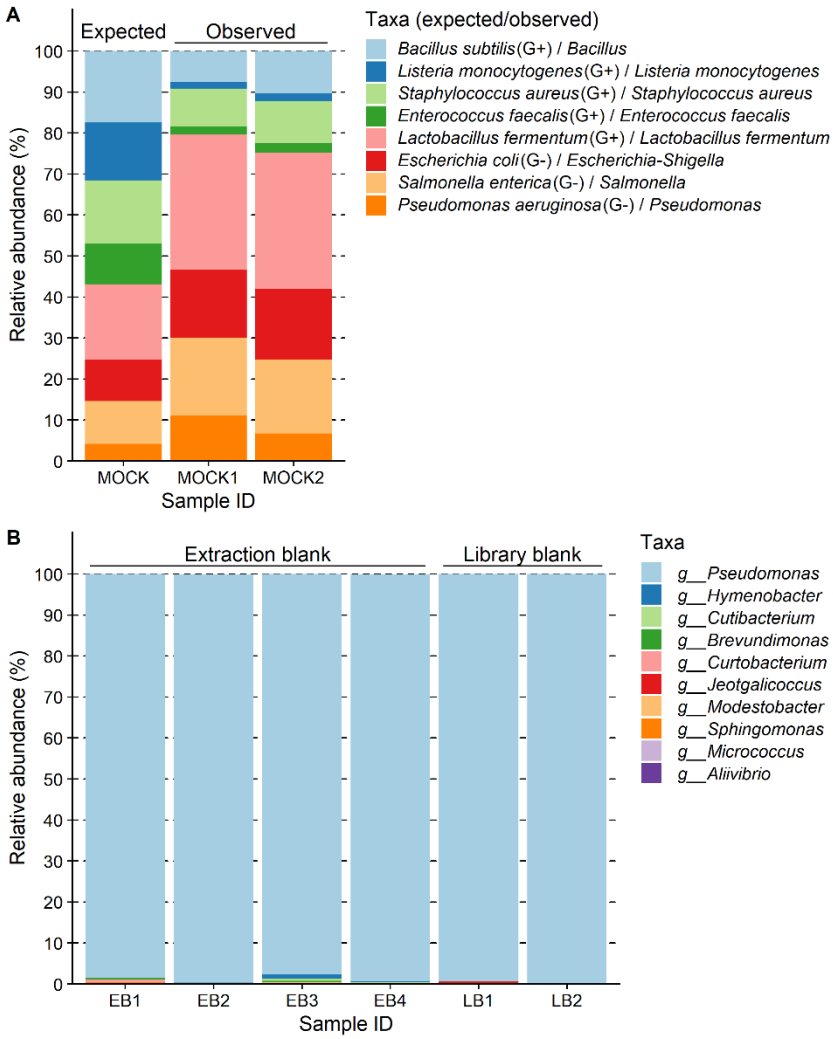

Fig. 6 Taxonomic composition of the mock (A) and negative controls (B). The highest taxonomic resolution was displayed for each taxon. g__, genus; EB, extraction blank; LB, library blank.

Paper 3

Research Article

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Total replacement of dietary fish meal with black soldier fly (*Hermetia illucens*) larvae does not impair physical, chemical or volatile composition of farmed Atlantic salmon (*Salmo salar* L.)

Leonardo Bruni,[a] Ikram Belghit,[b] Erik-Jan Lock,[b] Giulia Secci,[a] Cosimo Taiti[a] and Giuliana Parisi[a]*

Abstract

BACKGROUND: The aquafeed sector has been replacing conventional dietary ingredients with more economic and eco-friendly ingredients. Insects embody a promising alternative as a result of being highly nutritious and showing traits leading to a circular bioeconomy. Atlantic salmon (*Salmo salar* L.) at the sea-water stage were fed diets with a partial or complete substitution of fishmeal with meal of *Hermetia illucens* larvae reared on a media containing *Ascophyllum nodosum* mixed with organic wastes (60:40). The present study aimed to assess the quality of fillets by characterizing its physico-chemical traits with conventional and innovative methods, such as the proton transfer reaction-time of flight-mass spectrometer technique, allowing the analysis of samples at room temperature. Finally, steamed fillets underwent a consumer test to investigate the liking of consumers and their intention of re-consumption.

RESULTS: The main findings showed that a complete dietary substitution of fishmeal with *H. illucens* larvae meal did not impair the physico-chemical quality of *A. salmon* fillets. Notably, neutral *n*-3 polyunsaturated fatty acids (PUFA) slightly but significantly increased in the fillets of *A. salmon* fed *H. illucens*, also as a result of the additional fish oil present in the diets containing insect. The volatile organic profile was not altered by the different diets. The consumer-liking test revealed that Italian consumers appreciated the tested salmon irrespective of the administered feed.

CONCLUSION: Tailoring the insect fatty acid profile by rearing the larvae on a PUFA-rich substrate, coupled with a dietary modulation of the oily source, can successfully maintain or even increase the cardioprotective characteristics of fillets.

Supporting information may be found in the online version of this article.

Keywords: Atlantic salmon; black soldier fly; *Ascophyllum nodosum*; aquaculture; feed; PTR-ToF-MS

INTRODUCTION

The demand for animal protein is expected to increase in the near future. Because the production of conventional land-based farmed animals will unlikely be able to bear the increase in protein demand, other production animals are the focus of intensive research. Aquaculture and insect production are two of the most promising sectors. Their production is growing at a fast pace and they could positively contribute to the three pillars of sustainability (social, economic and environmental).[1,2] From a nutritional point of view, insects are suitable for human consumption, although cultural and psychological issues arise when they reach food market.[3] On the other hand, aquaculture products are appreciated much more by consumers worldwide for their nutritional [richness in *n*-3 polyunsaturated fatty acids (PUFA)] and sensory attributes. Moreover, an increase in income allows people to buy more expensive products, such as fish.[2]

Attention is required when it comes to the protein needs of farmed fish species. In western countries, these are mainly carnivores and their high protein needs are conventionally satisfied with fishmeal and vegetable protein sources, which are finite resources and also present economic, ecological and sustainable issues.[2] If insects are not yet appreciated in the food market, they represent a good alternative protein source for fish,

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Key words: Atlantic salmon, black soldier fly, *Ascophyllum nodosum*, aquaculture, feed, PTR-ToF-MS.

1. Introduction

The demand of animal protein is expected to increase in the near future. Since the production of conventional land-based farmed animals will unlikely be able to bear the increase in protein demand, other production animals are the object of intensive research. Aquaculture and insect production are two of the most promising sectors. Their production is growing at a fast pace and they could positively contribute to the three pillars of sustainability (social, economic and environmental).[1,2] From a nutritional point of view, insects are suitable for human consumption, but cultural and psychological issues arise when they reach food market.[3] On the other hand, aquaculture products are much appreciated by consumers worldwide for their nutritional (richness in n3 polyunsaturated fatty acids, PUFA) and sensory attributes. Moreover, an increase in the income allows people to buy more expensive products, such as fish.[2]

Attention must be paid when it comes to protein needs of farmed fish species. In western countries, these are mainly carnivores and their high protein needs are conventionally satisfied with fishmeal and vegetable protein sources, which are finite resources and present economic, ecological and sustainable issues.[2] If insects are not appreciated in the food market as yet, they represent a good alternative protein source for fish, as vastly acknowledged by numerous authors in the last years.[4-8] The European Commission has recently allowed the use of processed protein derived from seven species of farmed insects for aquaculture purposes.[9] The seven allowed species are: black soldier fly (*Hermetia illucens*, HI), 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*).

Salmonids fall back into the five most consumed fish category all around the world and are of great economical interest. The viability of including insects as feed ingredients for salmonids has been now ascertained, fish growth performance and digestibility pose little concern nowadays[4,10,11] and the first insect-based diets are now on the market. Current questions when including insects in diets for fish pertain to insect production costs, changes in fish microbiota and fish end-product quality. First, the industry of insect production is new and is constantly diminishing its production costs. Second, microbiota dwelling in the gut and on the fish surfaces is the target of a relatively new and rapidly growing research field, stretching from studies simply describing present microbes to studies on the function and role of microbiota. Finally, quality assessment is unavoidable when it comes to proposing to consumers new

products. Notoriously, different diets can have distinct effects on the final quality of the fish, meant as its set of physical, chemical and sensorial attributes. These are sternly sifted by the consumer perception, influencing her/his purchasing decision. For instance, reduction in PUFAn-3 altered color, texture, flavor[12] and it seems sensible that other sensorial attributes risk to jeopardize the customer acceptance of fish products.

At the origin of aroma and flavor of food, fish volatile profile might depend on fish growing conditions, in addition to cooking methods.[13] Borgogno and colleagues[14] found that including 20 and 40% of HI in diets for rainbow trout (*Oncorhynchus mykiss*) affected fillet sensory profile, as unraveled by a descriptive analysis and a temporal dominance of sensation methods made by trained panelists. However, sensory analyses are demanding in terms of sample quantity, time and staff. The technique proton transfer reaction-time of flight-mass spectrometer (PTR-ToF-MS) offers a rapid instrumental way to enquire the volatile profile (an important sensorial attribute) at room temperature and without sample manipulation, like mincing.[15]

The present study aimed to assess the fillet quality of Atlantic salmon (*Salmo salar* L.) at sea-water stage fed diets with different HI-inclusion levels. An analytical description of the raw fillets was obtained with physical and chemical conventional and innovative methods, such as PTR-ToF-MS technique. Finally, a consumer test on steamed samples of salmon was run to enquire liking judgements of consumers.

2. Materials and methods

2.1. Experimental diets, fish farming and sampling

The experimental protocol applied in this work was designed according to the ethical standards approved by the current European Directive 2010/63/EU[16] on the protection of animals used for scientific purposes.

The practical details of the feed ingredients and the feeding trials with A. salmon are reported in more detail elsewhere.[4] Briefly, post-smolt A. salmon were randomly distributed among 12 sea-cages (5×5×5 m; 125 m^3; 90 fish per cage, n=3 replicates) and were fed one of four isoproteic, isoenergetic and isolipidic diets for 16 weeks: a control diet (insect meal, IM_0, 0% of HI inclusion) and three diets with increasing substitution levels of the fishmeal with HI (33, 66 or 100%; IM_{33}, IM_{66} and IM_{100}, respectively). The larvae were grown on media partially containing *Ascophyllum nodosum* mixed with organic waste streams (60:40). The formulation and the analyzed composition of the experimental diets are shown in Table 1 (previously published in Belghit et al.[4]). When fish reached the commercial weight, they were percussively slaughtered, then filleted and frozen at -80 °C for transport and storage. Six fillets from each dietary group (two from each sea-cage) were allotted to physical and chemical analyses. Specifically, each fillet was thawed overnight at +4 °C prior to be cut into four parts corresponding

to cranial, caudal and two central regions. Firstly, the cranial and caudal portions of each fillet underwent the physical analyses described below, then, the same cranial and caudal portions of a single fillet were pooled and minced for chemical analyses. In parallel, on one of the two central portions of each fillet, the volatile organic compounds (VOCs) were determined. The other central portion was used for measurements that are not part of the present work. Other six fillets from each dietary group (two from each sea-cage) were allotted to consumer test.

2.2. Physical analyses

The pH was measured in two points of the cranial and caudal regions of each fillet by a pH-meter METTLER TOLEDO (Mettler-Toledo, Schwerzenbach, Switzerland). Fillet color measurement, expressed as L* (lightness), a* (redness), and b* (yellowness) indices,[17] was performed on two points of both cranial and caudal regions, using a Konica Minolta colorimeter (Chiyoda, Tokyo, Japan). Texture measurements were performed using a Zwick Roell® texturometer (Zwick GmbH & Co. KG, Ulm, Germany) equipped with a 1 kN load cell. A 4 × 4 cm section was cut from the cranial and caudal regions and underwent a two cycles compression test using a 10 mm diameter cylindrical probe (constant speed of 30 mm min^{-1}, 50% of total deformation). Test-Xpert2 by Zwick Roell® software version 3.0 was used for data collection and analysis. Hardness, cohesiveness, resilience, and adhesiveness were calculated as described by Veland and Torrissen.[18] Water holding capacity (WHC) was determined according to Hultmann and Rustad, 2002.[19] Briefly, the cranial and caudal portions of a single fillet were pooled and minced, then 2 g of minced flesh were sampled and inserted into plastic cylinders equipped with a filter net. The filled cylinders were centrifuged at 210 *g* for 5 min to remove free water. Hence, WHC was calculated as the difference between the initial gross weight of cylinders and their gross weight after centrifugation, divided by the water content of the minced sample. Water content was determined as described in the subsequent section. Each sample was analyzed in duplicate. The removed skin was analyzed by a manual caliper (Salmoiraghi, Milano, Italy) for the determination of the thickness in three points on both cranial and dorsal regions.

2.3. Proximate composition, total lipids and fatty acid composition

Moisture, crude protein (N × 6.25), and ash contents of minced flesh were determined following AOAC methods.[20] The total lipids of 2 g of each minced sample were extracted following the method proposed by Folch and co-authors.[21] Next, the polar and neutral fractions were obtained with the method proposed by Juaneda and Rocquelin,[22] with modifications by Loponte and colleagues.[23] The fatty acid (FA) profiles of polar and neutral lipids were analyzed by gas-chromatography using a Varian GC (Varian Inc., Palo Alto, CA, USA), after a base-catalyzed trans-esterification of the fatty acid.[24] The GC conditions were recovered from Secci et al.[25] Chromatograms were recorded with the Galaxie Chromatography Data System 1.9.302.952 computing integrator software (Varian Inc., Palo Alto, CA, USA). FAs were identified by comparing the FA methyl ester

(FAME) retention time with the Supelco 37 component FAME mix standard (Supelco, Bellefonte, PA, USA).

2.4. Volatile Organic Compounds (VOCs) analysis

VOC measurements of feeds and fillets were performed using a commercial PTR-ToF 8000 model (Ionicon Analytik GmbH, Innsbruck, Austria) in its standard configuration and using H_3O^+ as reagent ion for the proton-transfer reaction (see Ellis and Mayhew[26] for a detailed description of the technology). All mass spectra have been sampled ranging between m/z = 15 and m/z = 250. VOC measurements were performed using the system proposed by Colzi and co-authors.[27] Briefly, each fillet sample was put in a 3/4 L glass jar and topped with a special glass cap that allows the connection between the inlet of the PTR-ToF-MS, the headspace collecting and the zero-air generator. Sample's temperature was 18 °C. To avoid possible systematic errors, the apparatus was flushed with clean air for 1 min between the measurement of a sample and the next one. The signal intensity of each sample was recorded for 100 s, allowing the acquisition of 100 spectra (1 spectrum/1 sec). The spectra calibration was performed offline, using the following compounds: $H_3{}^{18}O^+$(water isotope, detected at m/z = 21.022); NO^+ (nitric oxide, detected at m/z = 29.999) and $C_3H_7O^+$ (acetone, detected at m/z = 59.049). To acquire the raw data (number of counts per second, cps), TofDaq software (Tofwerk AG, Switzerland) was used. Raw data were corrected to ppb according to the formula described by Lindinger and Jordan,[28] using a constant value for the reaction rate coefficient (kR = 2×10^{-9} cm^3/s). Afterwards, following the procedure described by Aprea et al.,[29] data were filtered by eliminating peaks imputable to water chemistry and to interfering ions, and also all peaks whose average concentrations were lower than a threshold of 0.25 ppbv, determined on empirical basis. Such filtering allowed discarding signals pertaining ions present in trace amounts. Finally, the tentative identification of VOCs provided by the tool was obtained comparing the measured masses with those reported in the instrument database and in the literature.[13,30,31]

2.5. Consumer test

Consumers' liking as well as their intention of re-consumption were analyzed by a blind product test. Eighty consumers, 40% males and 60% females, equally distributed into two age-ranges (25-45 and 46-65 years old), were recruited in Prato city and in the nearby area (Tuscany, Italy). The recruited people were familiar with the consumption of cooked salmon. Five sessions were conducted (4 sessions with 15 subjects and 1 session with 20 subjects, due to timetables) during the same day.

Before each session, fillets were thawed overnight at 4 °C, washed and accurately dried with paper towel. Fish bones and the caudal part were removed, then the residual part of the fillets was cut into 20-25 homogeneous portions of 17 ± 2 g each. Each portion was wrapped in aluminum foil with the skin on the bottom, then steam-cooked (1800D Thermostatic bath, Fratelli Galli, Milano, Italy) for 1.30 min (until 62 °C were reached at the core of a control sample) and

immediately presented to the consumers for tasting. No salt or oil were added to the portions. The samples were presented monadically, in a random order, and were identified by a three-digit code. Firstly, the consumers were asked to score their overall liking, then their liking for appearance, odor, flavor, and texture using a 9-points scale anchored with 1=extremely dislike, 9=extremely like).[32] The appropriateness of 8 sensory properties (color, visual texture, odor, flavor, salty intensity, tenderness, juiciness and fibrousness) were evaluated on a 5-points scale as suggested by the just-about-right scale.[33] At the end of the test, subjects had the opportunity to spontaneous comment about what they disliked. The intention of re-consumption was also collected for each type of product. Between two samples, consumers were asked to eat a cracker and drink half-glass of mineral water during a rest of 60 sec.

2.6. Statistical analyses

The statistical analyses of physical traits, proximate composition, FA profile (one-way ANOVA) and VOCs data (principal component analysis, PCA) were performed using the free software environment R,[34] with significance sets at $p=0.05$. All data are presented as means and pooled standard error of the mean (SEM), if not otherwise stated.

Consumer test data were analyzed using XL-stat software by a one-way ANOVA to determinate the effect of the HI inclusion in the diet on liking scores (significance sets at $p=0.05$). Penalty analysis was applied to appropriateness data to evaluate the decreases in the overall liking due to specific sensory attributes. Dunnet analysis was used as *post hoc* test to identify the significantly different ($p=0.05$) liking between the subjects who considered as appropriate a characteristic and those who did not.

3. Results

3.1. Physical traits

Results of the physical attributes of fillets showed no significant difference between the dietary groups (Table 2). No sharp trends could be highlighted for most of the parameters, however, taking lightness and yellowness into concern, the highest scores were noted in IM_{66} group, while this same group presented the lowest pH values.

3.2. Proximate composition

As shown in Table 2, moisture, ash and total lipids contents (g kg^{-1} of fillet) were not affected by the different dietary regimes. The highest crude protein content was found in IM_{66} fillets (209.2 g kg^{-1}), which differed ($p<0.05$) from IM_{100} group (194.8 g kg^{-1}); IM_0 and IM_{33} fillets showed intermediate values (196.8 and 206.7 g kg^{-1}, respectively).

3.3. Polar and neutral fatty acids

Polar FA profile of fillets (Table 3) differed between the dietary groups. The saturated FA (SFA) class was constant between the groups and was mainly composed by C16:0 (mean between groups: 14.156 g FA 100 g^{-1} polar FAME) and C18:0 (mean between groups: 4.382 g FA 100 g^{-1} polar FAME), while C12:0 content was irrelevant (mean between groups: 0.186 g FA 100 g^{-1} polar FAME). Monounsaturated FAs (MUFA) strongly decreased ($p<0.05$) following the dietary increase of IM inclusion in the diets and this result was mainly driven by the decrease in oleic acid content (C18:1n-9; 13.952 and 9.774 g FA 100 g^{-1} polar FAME in IM_0 and IM_{100}, respectively). PUFAn-6 content also decreased considerably ($p<0.01$) and this was mainly led by linoleic acid (C18:2n-6; 6.047 and 3.851 g FA 100 g^{-1} polar FAME in IM_0 and IM_{100}, respectively). Concerning the PUFAn-3 class, although ANOVA could not discriminate the groups based on the effect of the diets, a positive trend was apparent from IM_0 to IM_{100} groups, with a 15.6% increment in the total PUFA. The same increasing, although non-significant, trend was seen in eicosapentaenoic (EPA, C20:5n-3), docosapentaenoic (DPA, C22:5n-3) and docosahexaenoic (DHA, C22:6n-3) acid contents, while a subtle but significant decrease in α-linolenic acid (C18:3n-3) was revealed (3.407 and 2.435 g FA 100 g^{-1} polar FAME in IM_0 and IM_{100}, respectively; $p<0.01$).

In comparison to the polar fraction, larger modifications could be seen in the neutral FA profile (Table 4). Here, SFA and PUFAn-3 increased ($p<0.01$) with the increase of IM in the diets, while MUFA and PUFAn-6 decreased ($p<0.01$). The FA C12:0, C14:0 and C16:0 contributed to the overall increase in the SFA, while longer SFAs were constant between the dietary groups (C18:0) or present to a very small amount (C20:0, C22:0 and C24:0). The drop in the MUFA class was driven by C18:1 (IM_0: 35.361 g 100 g^{-1} of total neutral FAME; IM_{100}: 30.165 g 100 g^{-1} of total neutral FAME; $p<0.01$), but a significant increase in other MUFAs was also noted (C16:1n-7, C20:1n-9 and C22:1n-11; $p<0.01$). The most abundant n-6 FA was C18:2n-2, which decreased by 10.9% from IM_0 to IM_{100}. Finally, the increase in PUFAn-3 class was led by EPA and DHA, with a small but significant contribution of DPA; by contrast, α-linolenic acid decreased by 13.7% from IM_0 to IM_{100}.

3.4. Volatile organic compounds

In diets (Table S1) and fillets (Table 5), a total number of 37 different compounds was revealed using PTR-ToF-MS. The VOC contents of the fillets belonging to the four dietary groups were similar, as revealed by ANOVA and PCA (Figure S1). No manifest trends could be highlighted, except for CH_4O^+ (methanol), $C_3H_5^+$, $C_4H_7^+$, $C_5H_7^+$, $C_3H_7S^+$ (I.E. thietane) and $C_8H_{13}^+$, that seemed to increase following the increasing dietary HI meal inclusion, although no significant differences were observed. Computing different PCAs with reduced datasets, i.e., omitting some of the compounds found by PTR-ToF-MS considered to be irrelevant, did not help in descrying the four dietary groups (data not shown). VOCs of the four diets are shown in Table S1.

3.5. Consumer test

Results depicted in Table 6 highlighted that consumers' liking for the evaluated attributes was not affected by the administered diets. In addition, all the recorded mean values were favorable, i.e. equal or above 6. The appearance attributes (color and visual texture) were positively evaluated except for H_{66} and H_{100} color values, while the appropriateness percentages collected for the odor and flavor intensities of H_{66} group were the lowest (Odor: H_{66} = 63%; Flavor: H_{66} = 56%). On the other hand, H_{66} and H_{100} were the groups showing the highest adequacy of the intensity salty taste (H_{66} = 71%; H_{100} = 76%). It is also noteworthy that the H_{100} tenderness, juiciness and fibrousness were considered adequate only by 59%, 55% and 56% of consumers, respectively.

The penalty analysis allows to understand the liking drop of "not adequate" sensory characteristics; for this reason, Figure 1 (A, B, C, and D) synthesized the penalty analysis results.

The textural attributes led the lowering of the overall liking scores for all the experimental groups. However, while fibrousness represented a common weakness in IM_0, IM_{33}, IM_{66} and IM_{100} fillets, this was independent of feed formulation, tenderness and juiciness lowered the liking scores by 1.5 points in H_{66} and H_{100} fillets. The overall liking of the H_{100} group was also reduced because of the paler color of its fillets than those of the other groups.

The critical aspects mentioned were confirmed by the spontaneous dislike attributes generated by the consumers (Table 7). The results underlined how textural attributes of H_{100} were particularly disliked by more than the 50% of consumers, who described H_{100} texture "stringy". In addition, almost 20% of the participants slightly disliked the gustative items of H_{33} and H_{66}, having a light flavor and a salty taste. Despite this, at the end of the tasting session people were asked if they would intend to re-consume the fish, and the 83%, 78%, 73% and 78% of the people positively answered for IM_0, IM_{33}, IM_{66} and IM_{100} groups, respectively. Overall, the intention of re-consumption resulted to be high for all the experimental feeding groups.

4. Discussion

The main findings of the present study showed that a partial or complete substitution of FM protein with HI larvae meal in the diet did not impair the physico-chemical qualities of the salmon fillet. A description of the physical properties of Atlantic salmon fillets fed insect meal (IM) could not be found in the literature, while a few studies on other species are available, as shown below. Rainbow trout fed with diets where 0, 25 and 50% of the FM was substituted by a defatted HI meal did not show differences as regards texture (shear force) nor for lightness or redness indices, while a lower fillet yellowness index was noted in the group 50%.[14,30] In the present study, a dietary replacement of FM with HI larvae in the diets of salmon did not affect color, pH, WHC or the texture of the

flesh. Similarly, a partial replacement of FM with full-fat *T. molitor* larvae (TM, at 25 and 50% inclusion) did not exhibit any difference on the physiological traits of rainbow trout compared to the control group (where FM was the exclusive protein source).[35] However, in that study, the 50% group (inclusion levels of 500 g TM per kg diet) showed a higher redness index of the skin in comparison to the other groups.[35] The authors suggested that the effects observed might be due to the corn gluten meal inclusion in the diets.[35] In addition, the color parameter values of the skin, at the dorsal and ventral regions, were found similar in gilthead seabream (*Sparus aurata*) fed 25 and 50% full-fat *T. molitor* inclusion diets comparing to control diet containing FM and plant-based protein.[8] To sum up, changes in the physical properties of fish fed diets containing IM seem not to be tightly related to the dietary IM, but rather to the vegetable ingredients of the diet itself.

Our results on the proximate compositions of the A. salmon fillets were similar between treatments. This finding can be easily backed up by the similar growth parameters and apparent digestibility coefficients of the main macronutrients, as reported by Belghit and colleagues.[4] In fact, the fish of the present study were part of the same rearing trial as those of Belghit and colleagues.[4] As also revealed by several studies, the inclusion of HI in diets for fish seems not to impact on the proximate composition of fish.[5,14,36,37] In the present study, the difference found between the protein content in the fillets of A. salmon fed IM_{66} and IM_{100}, although statistically significant, is small (less than 2 g 100 g^{-1} fillet) and the protein contents were in general high and comparable to those fund in other studies performed in salmonids fed with IM diets.[11,14,35] Moreover, the difference between the protein contents of the two dietary groups would unlikely be important for consumers. Similarly, Dumas et al.[38] formulated four diets for rainbow trout where fishmeal was gradually replaced by HI (0, 25, 50 and 100%) and observed a significantly lower whole-body protein content in the 100% group as compared to the 0 and 25% groups; however, on the one hand, the administered diets were not strictly isoproteic and, certainly, the difference for the protein content between IM_{66} and IM_{100} groups was less than 2 g/whole fish.

The FA composition of both polar and neutral FAs in the fillets of A. salmon was affected by the different dietary treatments. In comparison to the neutral FAs, the proportions among the polar FAs were generally more stable between dietary groups. The main part of polar FAs is composed by phospholipids, which contribute to cell membrane structure, fluidity and selective permeability. To maintain a correct physiological status, the composition of cell membrane is generally modified depending on the life cycle stage of the fish and on the environmental factors. In the present study, more than half of the polar FAs was represented by n-3 PUFAs (~55%), followed by SFAs (~20%), MUFAs (~17%) and n-6 PUFAs (~7%). C12:0 is the dominant FA in HI larvae, containing up to 50% of total FA.[39,40] However, the content of this SFA was quite low in the fillet of salmon fed IM; although the IM used in the present study contained 30% C12:0 on total FA, the maximum inclusion of IM in the in IM_{100} diet was 150g/kg, thus, in total, the content of C12:0 in the diet was 2.3%. On the other hand, the dietary

effects on neutral FA profile was clearer. The FA composition of the A. salmon fillets generally reflected that of the diets. Similarly to the results obtained for the whole body of salmon fed with increasing inclusion of HI larvae,[4] the content of C12:0, C14:0 and C16:0 increased in the flesh of fish fed with the dietary HI inclusion, which is a consequence of the high content of these FAs in the HI larvae meal. The slight increase of neutral n-3 PUFAs in the fillets of A. salmon fed HI larvae was probably due to the additional fish oil present in the diets containing IM, rather than to the EPA and DHA contained in the HI larvae meal.[40] Specifically, EPA and DHA increased in IM_{66} and IM_{100}. This result shows that modulating the oily source can successfully maintain or even increase the cardioprotective characteristics of A. salmon fillets.

Since various volatile compounds are produced as a result of the FA oxidation, the VOC content is shaped by FA profile.[41] Nonetheless, our results showed that there were no apparent differences between the VOC contents in the fillet of salmon fed the four dietary treatments. The comparison between fillet VOCs and diet VOCs did not enable to see any relation between the two sample types. The research on the volatile profile of fish mainly enquired the differences between fresh and frozen-thawed product,[42] between wild and farmed fish, between different cooking methods,[13] or between fish fed different oily sources;[43] in the three abovementioned studies, significant differences were found between the different treatments, contrarily to our study where the diets did not model the VOCs; this could be due to the fact that the FA profile of our fillets was not deeply altered by the different dietary regimes. Differently, a study where rainbow trout was fed with diets where 0, 25 and 50% of the FM was substituted by a defatted HI meal did not highlight large differences as regards VOC content, with the exception of heptanal, that was lower in group 50% in comparison to 0%, and octanal, that was lower both in group 25% and in 50%.[30] However, the technique used in their study required minced samples and warming at 60 °C, while PTR-ToF-MS used in the present study analyzed an integral slice of salmon at room temperature, enabling the detection of those molecules that are native to the raw fish. Because of the recent tendency to eat raw fish, first and foremost A. salmon, its volatile profile is particularly interesting, and the analytical procedure should respect this need.

Numerous surveys concerning consumer attitude towards the use of insects as animal feed have been published in the recent years, mainly focusing on the stakeholders and consumers' acceptance in European countries.[44-47] At the same time, few studies evaluated the effect of insect-based feed on the sensory properties of fish fillets, obtaining different tendencies.[4,14,48] Indeed, few changes regarding the sensory attributes were highlighted by Belghit and colleagues[4] due to the increasing levels of HI meal in diets for A. salmon, while Borgogno and colleagues[14] asserted that HI inclusion in diets for rainbow trout significantly modified 12 out of 19 descriptors (texture, taste, aroma and flavor) used for the sensory profile analysis. However, the abovementioned studies wondered how the consumer would judge the fish, since variation in sensory properties may theoretically affect consumers' acceptance. From this starting point, the

consumer liking test on A. salmon fed with HI, assessed here for the first time, revealed that Italian consumers appreciated the tested salmon irrespective of the administered feed. Our findings agree with Sealey et al.,[48] who asked a group of 30 untrained panelists to indicate the odd sample in a set of three. In that test, people did not detect any significant differences in comparison to flesh of rainbow trout fed fishmeal or HI prepupae meal at graded inclusion levels.

On the other hand, the consumers of the present study pointed out some weaknesses in the HI groups related to color intensity, tenderness and juiciness. Noteworthily, these are the same parameters for which changes were detected by a trained panel testing baked fillets of A. salmon fed HI at increasing inclusion levels,[4] i.e. fish belonging to a trial parallel to the present's, but differently analyzed. For instance, color intensity decreased ($p<0.05$) and firmness tended to increase when increasing levels of HI substituted fishmeal in the diet for A. salmon.[4]

Color is deeply affected by the cooking process. In our case, as fillet samples were wrapped in aluminum foil, it can be thought that liquid removal was prevented, thus forming a pale layer on the sample surface. The light color highlighted in IM_{66} and IM_{100} samples, in comparison to the other groups, could be explained by a lower water holding capacity (WHC) of the cooked samples (whose study was outside the framework of the present paper). Given the hypothetical lower WHC of the cooked samples, tenderness and juiciness would also possibly diminish. WHC, as well as textural attributes, such as tenderness and juiciness, depend on the protein matrix. A recent result by Iaconisi and colleagues[49] partly goes the same way as our hypothesis, finding that the amino acid profile of fillets was affected by feeding rainbow trout with *Tenebrio molitor* larvae. Nonetheless, the perceived differences and spontaneously dislike attributes generated did not seriously compromise the overall liking for the products of the present trial. Further studies on the effect of dietary insects on protein component seem desirable.

5. Conclusions

In conclusion, the present study shows that HI larvae meal seemed to be a promising protein source for a partial or complete replacement of fishmeal in the diet for A. salmon of a commercially relevant size. Only minor effects on protein, fatty acid composition and fillet quality were detected replacing up to 100% of fishmeal with HI larvae meal. The study demonstrated the possibility of successfully maintaining or even increasing the cardioprotective characteristics of fillets from A. salmon fed with this new protein source, tailoring the insect FA profile by including macroalgae in the larval rearing substrate and with a modulation of the oily source in the aquafeed formulation. The consumer test on steamed fillets demonstrated that samples were evenly appreciated irrespective of the dietary group, although small weaknesses were observed in the fillets of fish fed HI larvae meal, which were related to color intensity and textural

attributes. It would be interesting to investigate if providing information about the feeding strategy provided during the tasting session could affect the consumers' liking.

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Table 1. Formulation and proximate composition of the four experimental diets (IM_0, IM_{33}, IM_{66} and IM_{100}) fed to Atlantic salmon (*Salmo salar* L.) (previously published in Belghit et al., 2019).

	IM_0	**IM_{33}**	**IM_{66}**	**IM_{100}**
Ingredients (%)				
Fishmeal LT94	10	6.67	3.33	0.0
Insect meal	0.0	4.91	9.84	14.75
Soy protein concentrate	25	25	25	25
Corn gluten meal	7.5	7.5	7.5	7.5
Wheat gluten meal	3.35	4.51	5.7	6.88
Pea protein concentrate 55	8.8	6.8	4.8	2.84
Fish oil	10.18	11.70	13.23	14.76
Rapeseed oil	20.95	18.86	16.79	14.73
Binder	12.32	12.08	11.72	11.24
Additives	1.89	1.96	2.1	2.29
Yttrium	1.0	1.0	1.0	1.0
Proximate analysis				
DM (%)	93	93	94	95
Crude protein (%)	38	38	39	39
Crude lipids (%)	29	29	29	29
Ash (%)	4.6	4.6	4.5	4.5
Carbohydrates (%)	11.6	11.5	11.5	11.4
Gross energy (MJ/kg)	24.6	24.9	24.8	25.0
TBARS (nmol/g)	3.0	3.4	4.2	4.9

TBARS: thiobarbituric acid-reactive substances.

Table 2. Physical parameters and chemical composition (g kg^{-1} of fillet) of the fillets from Atlantic salmon (*Salmo salar* L.) fed the four experimental diets (IM_o, IM_{33}, IM_{66} and IM_{100}). Values are means and pooled standard error of the mean (SEM) (n=6).

	Diets				SEM[†]	*p-value*[‡]
	IM_o	**IM_{33}**	**IM_{66}**	**IM_{100}**		
Physical parameters						
pH	6.24	6.21	6.08	6.19	0.030	*ns*
WHC (%)	91.669	92.686	91.947	90.802	0.359	*ns*
Color						
L*	45.13	45.88	46.14	45.93	0.475	*ns*
a*	10.97	11.20	11.49	11.51	0.411	*ns*
b*	11.85	12.09	12.56	11.68	0.382	*ns*
Skin Thickness, µm	516	466	541	483	15	*ns*
Texture						
Hardness, N	4.82	5.15	4.93	5.11	0.18	*ns*
Cohesiveness	0.22	0.23	0.24	0.21	0.01	*ns*
Resilience	0.03	0.03	0.02	0.02	0.00	*ns*
Adhesiveness	1.53	1.48	1.55	1.46	0.18	*ns*
Chemical composition						
Water	641.6	632.4	626.0	627.5	0.311	*ns*
Ash	13.2	13.4	13.3	13.7	0.018	*ns*
Crude protein	196.8^{ab}	206.7^{ab}	209.2^{a}	194.8^{b}	0.203	*
Total lipids	136.1	130.9	137.0	138.8	0.421	*ns*

WHC: water holding capacity; L*: lightness; a*: redness index; b*: yellowness index.

[†] SEM: standard error of the mean.

[‡] ns: not significant ($p>0.05$); a, b, c as superscript letters are significant different means at * $p<0.05$; ** $p<0.01$.

Table 3. Fatty acid composition of the polar lipid fraction (g FA 100 g^{-1} polar FAME) of the fillets from Atlantic salmon (*Salmo salar* L.) fed the four experimental diets (IM_0, IM_{33}, IM_{66} and IM_{100}).

	Diets				SEM[†]	*p-value*[‡]
	IM_0	**IM_{33}**	**IM_{66}**	**IM_{100}**		
C12:0	0.135^{b}	0.168^{ab}	0.211^{ab}	0.228^{a}	0.012	*
C14:0	1.089	1.197	1.281	1.351	0.038	*ns*
C15:0	0.190	0.180	0.162	0.168	0.004	*ns*
C16:0	14.299	14.371	13.948	14.001	0.295	*ns*
C16:1n-9	0.190	0.170	0.158	0.146	0.006	*ns*
C16:1n-7	0.692	0.756	0.773	0.738	0.232	*ns*
C17:0	0.358	0.364	0.293	0.339	0.010	*ns*
C18:0	4.452	4.270	4.519	4.287	0.085	*ns*
C18:1n-9	13.952^{a}	13.128^{a}	11.684^{ab}	9.774^{b}	0.458	*
C18:1n-7	1.649	1.582	1.480	1.292	0.045	*ns*
C18:2n-6	6.047^{a}	5.275^{ab}	4.543^{bc}	3.851^{c}	0.214	**
C18:3n-4	0.149^{b}	0.173^{ab}	0.194^{a}	0.184^{a}	0.005	**
C18:3n-3	3.407^{a}	3.042^{ab}	2.770^{b}	2.435^{c}	0.084	**
C18:4n-3	0.374	0.345	0.370	0.359	0.008	*ns*
C18:4n-1	0.146^{b}	0.172^{b}	0.212^{a}	0.204^{a}	0.007	**
C20:1n-9	1.377	1.369	1.193	1.079	0.052	*ns*
C20:2n-6	0.722^{a}	0.710^{a}	0.634^{ab}	0.511^{b}	0.025	*
C20:3n-6	0.730^{a}	0.664^{ab}	0.589^{ab}	0.512^{b}	0.025	*
C20:4n-6	0.764^{c}	0.831^{bc}	0.914^{ab}	0.999^{a}	0.021	**
C20:3n-3	0.384^{a}	0.353^{a}	0.345^{a}	0.265^{b}	0.011	**
C20:4n-3	1.115	1.153	1.217	1.080	0.022	*ns*
C20:5n-3, EPA	9.601	9.893	10.686	11.023	0.239	*ns*
C22:1n-11	0.754	0.731	0.553	0.524	0.041	*ns*
C22:5n-6	0.501	0.304	0.270	0.285	0.040	*ns*
C22:5n-3, DPA	3.348	4.061	3.659	4.373	0.216	*ns*
C22:6n-3, DHA	32.239	33.445	36.090	38.775	0.928	*ns*
ΣSFA	20.958	20.934	20.707	20.667	0.392	*ns*
ΣMUFA	19.088^{a}	18.171^{ab}	16.219^{ab}	13.913^{b}	0.615	*
ΣPUFAn-6	8.828^{a}	7.924^{ab}	7.070^{bc}	6.273^{c}	0.251	**
ΣPUFAn-3	50.583	52.366	55.296	58.469	1.103	*ns*

The following FAs were utilized for calculating the classes of FAs but they are not listed because below 0.15% of the total FAME: C13:0, C14:0 iso, C14:1n-5, C15:0 iso, C15:0 anteiso, C16:3n-4, C17:1, C16:4n-1, C16:2n-4, C16:0 iso, C18:3n-6, C18:2n-4, C20:0, C20:1n-11, C20:1n-7, C22:1n-9, C22:1n-7, C22:2n-6, C21:5n-3, C22:4n-6, C22:0, C24:0.

[†] SEM: standard error of the mean.

[‡] ns: not significant (p>0.05); a, b, c as superscript letters are significant different means at * p<0.05; ** p<0.01.

Table 4. Fatty acid composition of the neutral lipid fraction (g FA 100 g^{-1} neutral FAME) of the fillets from Atlantic salmon (*Salmo salar* L.) fed the four experimental diets (IM_0, IM_{33}, IM_{66} and IM_{100}).

	Diets				SEM†	*p-value*‡
	IM_0	**IM_{33}**	**IM_{66}**	**IM_{100}**		
C12:0	0.081^{d}	0.516^{c}	1.124^{b}	1.705^{a}	0.129	**
C14:0	1.850^{d}	2.192^{c}	2.466^{b}	2.740^{a}	0.07	**
C16:0	8.326^{b}	8.637^{a}	8.872^{ab}	8.922^{a}	0.076	*
C16:1n-9	0.181^{a}	0.171^{ab}	0.166^{b}	0.166^{b}	0.002	*
C16:1n-7	1.776^{d}	1.948^{c}	2.110^{b}	2.268^{a}	0.040	**
C16:2n-4	0.241^{d}	0.268^{c}	0.316^{b}	0.347^{a}	0.009	**
C17:0	0.188	0.194	0.201	0.198	0.002	*ns*
C18:0	2.879	2.907	2.951	2.807	0.033	*ns*
C18:1n-9	35.361^{a}	33.636^{b}	31.328^{c}	30.165^{d}	0.423	**
C18:1n-7	2.371^{a}	2.314^{b}	2.239^{c}	2.203^{c}	0.015	**
C18:2n-6	12.752^{a}	12.074^{b}	11.502^{c}	11.356^{c}	0.012	**
C18:3n-3	5.189^{a}	4.831^{b}	4.622^{bc}	4.478^{c}	0.062	**
C18:4n-3	0.915^{b}	0.878^{b}	0.940^{ab}	1.009^{a}	0.013	**
C18:4n-1	0.190^{c}	0.221^{b}	0.259^{a}	0.277^{a}	0.008	**
C20:0	0.296	0.308	0.302	0.296	0.004	*ns*
C20:1n-11	0.295	0.315	0.336	0.297	0.007	*ns*
C20:1n-9	5.204^{c}	5.522^{b}	5.750^{ab}	5.808^{a}	0.058	**
C20:2n-6	1.269^{a}	1.282^{a}	1.206^{ab}	1.108^{b}	0.020	**
C20:3n-6	0.393^{ab}	0.392^{a}	0.339^{ab}	0.315^{b}	0.012	*
C20:4n-6	0.144^{b}	0.155^{b}	0.172^{a}	0.176^{a}	0.003	**
C20:3n-3	0.569^{a}	0.539^{a}	0.514^{ab}	0.452^{b}	0.013	**
C20:4n-3	1.085	1.140	1.194	1.182	0.020	*ns*
C20:5n-3, EPA	3.128^{c}	3.347^{bc}	3.544^{ab}	3.683^{a}	0.057	**
C22:1n-11	4.417^{d}	4.853^{c}	5.413^{b}	5.696^{a}	0.106	**
C22:1n-9	0.677^{b}	0.703^{ab}	0.715^{a}	0.707^{ab}	0.005	*
C21:5n-3	0.204^{c}	0.228^{b}	0.260^{a}	0.277^{a}	0.010	**
C22:5n-6	0.351	0.245	0.290	0.175	0.036	*ns*
C22:5n-3, DPA	1.771^{b}	2.094^{a}	2.155^{a}	2.245^{a}	0.045	**
C22:6n-3, DHA	6.547^{b}	6.613^{b}	7.143^{a}	7.352^{a}	0.089	**
ΣSFA	14.071^{d}	15.263^{c}	16.424^{b}	17.171^{a}	0.260	**
ΣMUFA	50.478^{a}	49.670^{b}	48.272^{c}	47.519^{d}	0.254	**
ΣPUFAn-6	15.163^{a}	14.395^{b}	13.734^{c}	13.333^{c}	0.152	**
ΣPUFAn-3	19.407^{b}	19.670^{b}	20.371^{a}	20.677^{a}	0.133	**

The following FAs were used for calculating the classes of FAs but they are not listed because below 0.15% of the total FAME: C13:0, C14:0 iso, C14:1n-5, C15:0 iso, C15:0 anteiso, C15:0, C16:0 iso, C16:3n-4, C17:1, C16:4n-1, C18:2n-4, C18:3n-6, C18:3n-4, C20:1n-7, C22:0, C22:1n-7, C22:2n-6, C22:4n-6, C24:0.

† SEM: standard error of the mean.

‡ ns: not significant (p>0.05); a, b, c as superscript letters are significant different means at * p<0.05; ** p<0.01.

Table 5. List of measured VOCs in the fillets from Atlantic salmon (*Salmo salar* L.) fed the four experimental diets (IM_0, IM_{33}, IM_{66} and IM_{100}) and their tentative identification. Values are expressed as group means (ppb) ± standard deviation.

m/z[†]	Sum formula	Concentration				Tentative identification	p-value[‡]
		IM_0	IM_{33}	IM_{66}	IM_{100}		
15.03	CH_3^+	9.31±3.43	8.26±4.94	6.49±5.11	8.57±4.28	Unidentified	ns
27.02	$C_2H_3^+$	9.49±0.50	8.45±4.21	7.26±3.93	8.49±3.61	Acetylene[1]	ns
31.02	CH_3O^+	6.56±1.67	4.90±1.87	4.58±3.09	6.13±3.06	Formaldehyde	ns
33.03	CH_4O^+	5.72±3.61	6.47±3.90	6.58±2.66	7.76±3.84	Methanol	ns
41.04	$C_3H_5^+$	5.44±2.03	5.92±3.46	6.31±3.84	6.75±1.80	Unidentified	ns
43.02	$C_2H_3O^+$	12.15±5.85	13.67±10.41	8.11±4.95	12.20±6.29	Unidentified	ns
43.05	$C_3H_7^+$	3.28±1.55	3.21±1.92	3.01±1.33	3.04±0.74	Unidentified	ns
45.03	$C_2H_5O^+$	49.91±23.81	42.66±15.48	40.20±20.67	53.53±31.36	Acetaldehyde	ns
47.05	$C_2H_7O^+$	0.28±0.20	0.40±0.16	0.25±0.15	0.30±0.15	Ethanol	ns
55.05	$C_4H_7^+$	2.08±1.41	2.47±1.95	2.74±2.26	2.89±1.66	Unidentified	ns
57.03	$C_3H_5O^+$	0.44±0.58	0.69±0.99	0.31±0.46	0.27±0.47	Unidentified	ns
57.07	$C_4H_9^+$	1.74±1.28	1.74±1.03	2.00±0.76	1.71±0.62	Unidentified	ns
59.05	$C_3H_7O^+$	14.88±2.55	13.38±3.94	15.66±2.34	16.90±3.34	Propanal[3]	ns
61.03	$C_2H_5O_2^+$	1.71±2.36	3.75±5.64	0.79±1.76	2.99±3.59	Acetic acid	ns
67.05	$C_5H_7^+$	0.47±0.36	0.55±0.21	0.62±0.30	0.67±0.22	Unidentified	ns
69.07	$C_5H_9^+$	0.52±0.87	0.77±1.04	1.23±1.56	0.99±0.85	Unidentified	ns
73.06	$C_4H_9O^+$	0.49±0.16	0.65±0.26	0.47±0.12	0.69±0.23	2-Methylpropanal[4]	ns
75.03	$C_3H_7S^+$	14.68±4.85	17.43±8.17	16.21±8.99	19.17±10.51	I.E. Thietane	ns
77.02	$C_2H_5O_3^+$	1.96±0.74	2.38±1.25	2.07±1.49	2.76±1.83	Unidentified	ns
79.05	$C_6H_7^+$	0.31±0.22	0.16±0.23	0.14±0.21	0.36±0.36	Unidentified	ns
81.06	$C_5H_7N^+$	0.34±0.25	0.42±0.15	0.62±0.35	0.45±0.31	1-Methyl-1H-pyrrole[4]	ns
83.09	$C_6H_{11}^+$	0.15±0.29	0.25±0.37	0.51±0.56	0.43±0.45	Unidentified	ns
91.06	$C_7H_7^+$	0.65±0.48	0.33±0.47	0.28±0.30	0.73±0.69	Unidentified	ns
93.07	$C_7H_9^+$	0.64±0.25	0.52±0.15	0.49±0.09	0.80±0.51	Unidentified	ns
95.02	$C_2H_7O_2S^+$	0.14±0.16	0.19±0.17	0.12±0.16	0.18±0.22	Methylsulfonylethane	ns
105.1	$C_8H_9^+$	0.42±0.29	0.23±0.32	0.16±0.23	0.50±0.43	Unidentified	ns
107.1	$C_7H_7O^+$	0.69±0.48	0.45±0.46	0.46±0.27	0.92±0.67	Benzaldehyde[2]	ns
109.1	$C_8H_{13}^+$	0.32±0.25	0.35±0.36	0.40±0.37	0.57±0.32	Unidentified	ns
121.1	$C_9H_{13}^+$	0.59±0.39	0.53±0.23	0.56±0.14	0.79±0.48	Unidentified	ns

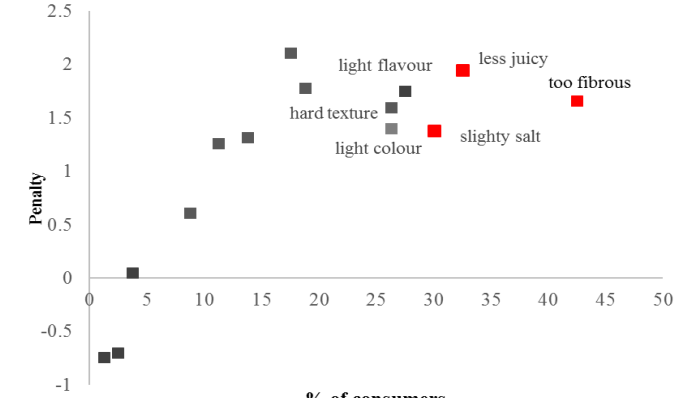
[1] Instrument database.
[2] Nieva-Echevarría et al. (2017),[31] doi.org/10.1016/j.foodres.2017.06.043.
[3] Mancini et al. (2017),[30] doi.org/10.1017/S1751731117003421.
[4] Nieva-Echevarría et al. (2018),[13] doi.org/10.1016/j.foodres.2017.10.029.
[†] m/z: mass to charge ratio
[‡] ns: not significant (p>0.05).

Table 6. Consumers' liking expressed for Atlantic salmon (*Salmo salar* L.) fed the four experimental diets (IM_0, IM_{33}, IM_{66} and IM_{100}). Values are expressed as group means ± standard deviation.

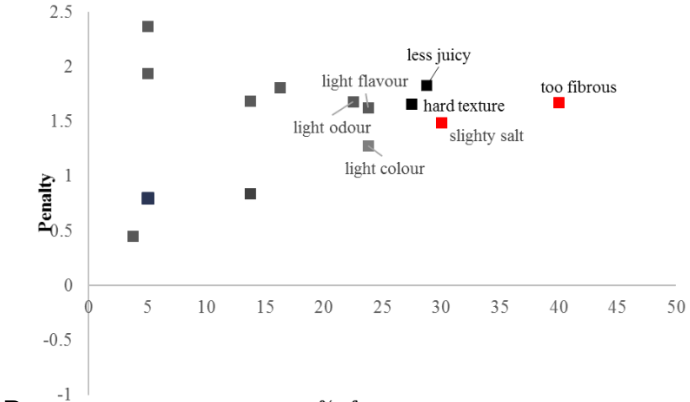
	Diets				***p-value***
	IM_0	**IM_{33}**	**IM_{66}**	**IM_{100}**	
Overall	6.90 ± 1.56	6.80 ± 1.46	6.55 ± 1.80	6.79 ± 1.53	*0.513*
Appearance	6.70 ± 1.42	6.95 ± 1.25	6.47 ± 1.62	6.84 ± 1.39	*0.101*
Odor	6.60 ± 1.41	6.81 ± 1.33	6.36 ± 1.73	6.78 ± 1.38	*0.118*
Flavor	6.83 ± 1.43	6.75 ± 1.55	6.34 ± 1.78	6.86 ± 1.38	*0.067*
Texture	6.41 ± 1.56	6.34 ± 1.61	6.20 ± 1.69	6.39 ± 1.62	*0.803*

Table 7. Amount of the spontaneous dislike aspects highlighted by the consumers after the blind product analysis of Atlantic salmon (*Salmo salar*) fed the four experimental diets (IM_0, IM_{33}, IM_{66} and IM_{100}).

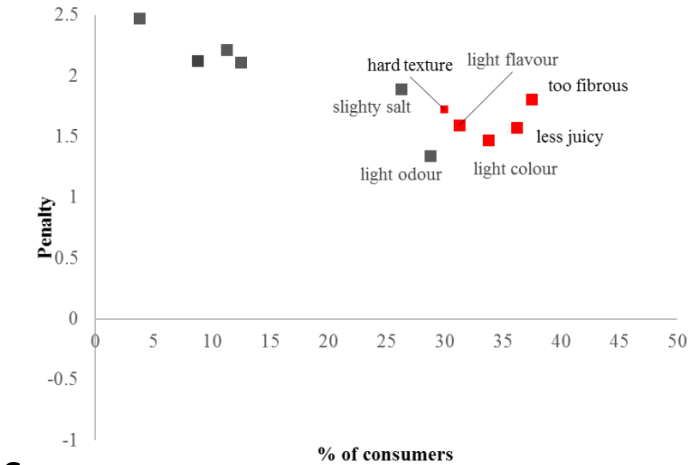
	Diets			
	IM_0	**IM_{33}**	**IM_{66}**	**IM_{100}**
light color	3	3	7	6
dark color	0	0	1	0
white cover	2	1	1	1
not appealing	1	1	0	0
Total visual attributes	*6*	*5*	*9*	*7*
odor	1	3	0	0
light odor	1	0	1	2
strong odor	0	4	2	0
Total olfactive attributes	*2*	*7*	*3*	*2*
flavor	1	2	1	1
light flavor	9	8	8	5
strong flavor	0	3	2	0
bitter	1	2	1	0
slightly salt	0	3	5	1
metallic flavor	0	1	0	0
earthy flavor	0	0	0	1
Total gustative attributes	*11*	*19*	*17*	*8*
watery	2	1	3	0
soft texture	3	1	3	1
texture	4	4	1	1
stringy texture	19	16	17	40
compact	4	1	3	5
fat perception	0	0	1	0
Total textural attributes	*32*	*23*	*28*	*47*


light flavour
less juicy
too fibrous
hard texture
slighty salt
light colour
Penalty
% of consumers

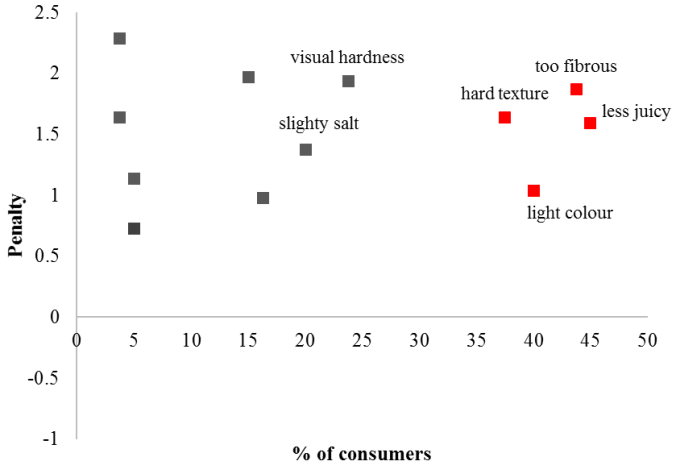
A


less juicy
light flavour
too fibrous
hard texture
light odour
slighty salt
light colour
Penalty
% of consumers

B


hard texture
light flavour
too fibrous
slighty salt
less juicy
light odour
light colour
Penalty
% of consumers

C



D

Figure 1. Penalty analysis assessed on fillets from Atlantic salmon (*Salmo salar* L.) fed the four experimental diets (IM_0, IM_{33}, IM_{66} and IM_{100}: A, B, C and D, respectively). Red squares are significant ($p<0.05$) sensory attributes. Only the penalties over 20% has been reported in the graphs.

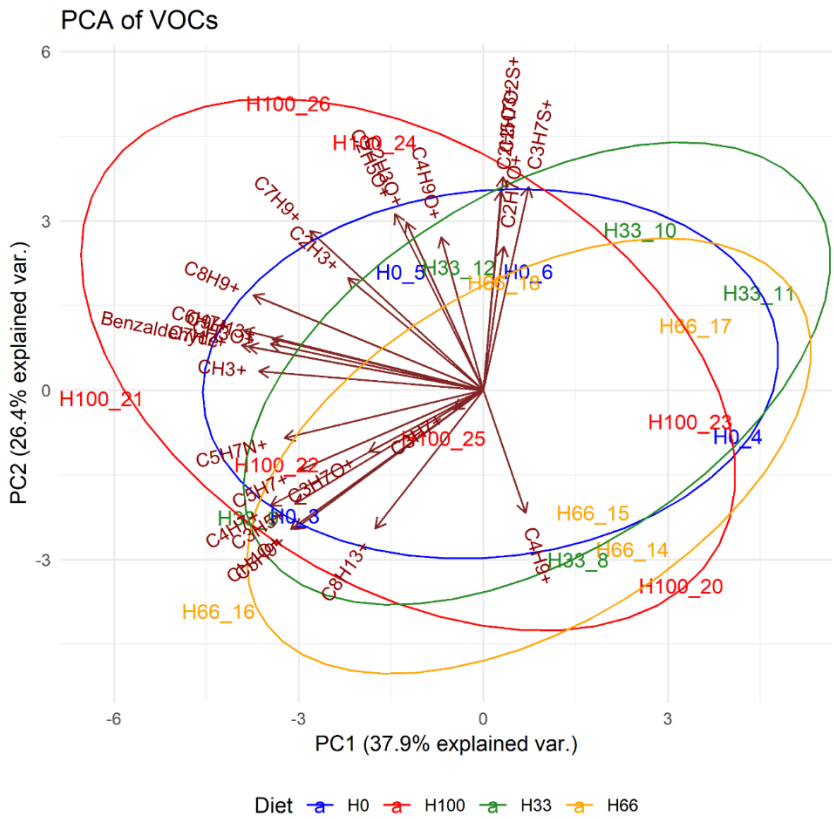


Figure S1. Principal components analysis of the volatile organic compound profile of fillets of Atlantic salmon (*Salmo salar* L.) fed the four experimental diets (IM_0, IM_{33}, IM_{66} and IM_{100}).

Table S1. List of measured VOCs in the four experimental diets for Atlantic salmon (*Salmo salar* L.) (expressed as mean concentration ppb ± sd) and their tentative identification.

m/z[†]	Sum formula	Concentration				Tentative identification	p-value[‡]
		IM_0	IM_{33}	IM_{66}	IM_{100}		
15.03	CH_3^+	58.81 ± 28.65	36.62 ± 21.53	51.82±37.26	18.28 ± 2.25	Acetic acid[1]	ns
27.02	$C_2H_3^+$	12.76 ± 1.97	14.03 ± 0.34	18.53±9.64	11.63 ± 1.77	Acetylene[1]	ns
31.02	CH_3O^+	4.36 ± 0.30	4.22 ± 0.13	6.48±2.69	5.47 ± 0.92	Formaldehyde	ns
33.03	CH_4O^+	45.37 ± 33.45	25.85 ± 15.81	41.93±37.84	8.17 ± 1.28	Methanol	ns
41.04	$C_3H_5^+$	39.13 ± 2.47	42.13 ± 16.74	43.66±6.92	39.82 ± 0.43	Unidentified	ns
43.02	$C_2H_3O^+$	94.78 ± 78.06	83.38 ± 60.24	102.88±81.30	38.39 ± 1.07	Unidentified	ns
43.05	$C_3H_7^+$	11.67 ± 3.75	12.59 ± 0.67	15.05 ± 6.80	10.91 ± 0.59	Unidentified	ns
45.03	$C_2H_5O^+$	8.79 ± 1.56	9.69 ± 1.50	15.45 ± 9.17	9.01 ± 0.08	Acetaldehyde	ns
47.05	$C_2H_7O^+$	0.86 ± 0.80	0.83 ± 0.74	1.67 ± 1.94	0.40 ± 0.11	Ethanol	ns
49.01	CH_5S^+	0 ± 0	0.16 ± 0.22	0.37 ± 0.52	0 ± 0	Methanethiol[2]	ns
54.03	$C_3H_4N^+$	4.46 ± 0.19	4.90 ± 1.84	4.36 ± 0.69	3.48 ± 0.03	Propanenitrile	ns
55.05	$C_4H_7^+$	104.10 ± 6.36	115.72 ± 43.68	112.00±20.36	86.84 ± 1.50	Unidentified	ns
57.03	$C_3H_5O^+$	6.27 ± 3.23	7.39 ± 3.78	9.58 ± 6.80	4.91 ± 0.12	Unidentified	ns
57.07	$C_4H_9^+$	3.67 ± 0.72	3.99 ± 0.39	4.53 ± 1.13	3.78 ± 0.14	Unidentified	ns
59.05	$C_3H_7O^+$	6.96 ± 3.32	7.73 ± 4.72	15.04±13.00	6.19 ± 0.95	Propanal[3]	ns
61.03	$C_2H_5O_2^+$	86.57 ± 85.04	75.86 ± 68.65	92.89±85.86	25.77 ± 6.94	Acetic acid	ns
63.03	$C_2H_7S^+$	0.41 ± 0.58	0.42 ± 0.59	0.59±0.84	0 ± 0	Ethanethiol[2]	ns
67.05	$C_5H_7^+$	1.72 ± 0.12	1.88 ± 0.71	1.91 ± 0.02	2.26 ± 0.39	Unidentified	ns
69.07	$C_5H_9^+$	34.38 ± 1.92	34.75 ± 14.33	32.00 ± 3.51	27.06 ± 0.08	Unidentified	ns
71.05	$C_4H_7O^+$	2.04 ± 0.32	2.24 ± 0.10	2.50 ± 0.54	2.58 ± 0.53	Unidentified	ns
73.06	$C_4H_9O^+$	2.72 ± 1.91	3.01 ± 2.11	3.44 ± 2.87	1.33 ± 0.56	2-Methylpropanal[4]	ns
75.03	$C_3H_7S^+$	14.66 ± 15.41	17.25 ± 18.81	19.37±19.28	11.85 ± 8.90	I.E. Thietane	ns
77.02	$C_2H_5O_3^+$	1.83 ± 1.20	2.05 ± 1.29	1.87 ± 1.24	2.14 ± 1.06	Unidentified	ns
79.05	$C_6H_7^+$	4.17 ± 0.12	4.53 ± 1.50	4.12 ± 0.56	3.91 ± 0.46	Unidentified	ns
81.07	$C_6H_9^+$	3.37 ± 0.13	3.46 ± 1.249	3.56 ± 0.28	3.80 ± 0.40	Unidentified	ns
83.09	$C_6H_{11}^+$	1.72 ± 0.18	1.97 ± 0.65	2.84 ± 0.59	2.37 ± 0.49	Unidentified	ns
87.08	$C_5H_{11}O^+$	0.20 ± 0.28	0.38 ± 0.11	0.59 ± 0.32	0.24 ± 0.35	Pentanal[3]/3-Methylbutanal[2,4]/ 1-penten-3-ol[3]	ns
89.06	$C_4H_9O_2^+$	2.45 ± 2.00	2.77 ± 2.29	2.79 ± 2.72	0.80 ± 0.10	I.E. Acetoin/cis-Butene-1,4-diol/Butyric acid	ns
91.06	$C_7H_7^+$	1.27 ± 0.40	1.49 ± 0.90	1.15 ± 0.27	1.73 ± 0.69	Unidentified	ns
93.07	$C_7H_9^+$	1.26 ± 0.19	1.29 ± 0.06	1.12 ± 0.37	1.64 ± 0.02	Unidentified	ns
95.07	$C_6H_9N^+$	2.97 ± 0.24	3.75 ± 1.06	3.11 ± 0.30	2.53 ± 0.04	1-Ethylpyrrole[4]	ns
95.09	$C_7H_{11}^+$	2.54 ± 0.20	2.87 ± 0.70	3.06 ± 0.68	2.77 ± 0.21	Unidentified	ns
96.08	$C_6H_{10}N^+$	44.36 ± 10.56	43.98 ± 10.49	40.05±11.23	32.93 ± 4.58	1-Ethylpyrrolidine	ns
97.09	$C_3H_{13}O_3^+$	3.58 ± 0.78	3.66 ± 0.94	3.63±0.98	2.98 ± 0.39	Unidentified	ns

103.1	$C_5H_{11}O_2^+$	0.78 ± 0.48	1.00 ± 0.67	1.01 ± 0.82	0.49 ± 0.00	Unidentified	ns
105.1	$C_8H_9^+$	0.49 ± 0.16	0.63 ± 0.29	0.50 ± 0.09	0.83 ± 0.38	Fragment	ns
107.1	$C_7H_7O^+$	1.50 ± 0.10	1.83 ± 0.58	1.60 ± 0.16	2.23 ± 0.12	Benzaldehyde[2]	ns
109.1	$C_8H_{13}^+$	1.47 ± 0.19	1.87 ± 0.20	2.32 ± 0.47	2.19 ± 0.12	Unidentified	ns
111.1	$C_7H_{11}O^+$	0.57 ± 0.11	0.50 ± 0.04	0.56 ± 0.12	0.84 ± 0.05	2,4-heptadienal[3]	ns
112.1	$C_7H_{14}N^+$	1.41 ± 0.39	1.36 ± 0.23	1.28 ± 0.36	1.23 ± 0.14	Ethylcyclopentane	ns
121.1	$C_9H_{13}^+$	0.60 ± 0.05	0.77 ± 0.17	0.62 ± 0.03	0.80 ± 0.03	Unidentified	ns
123.1	$C_9H_{15}^+$	0 ± 0	0.13 ± 0.19	0.19 ± 0.26	0.13 ± 0.19	Unidentified	ns
125.1	$C_8H_{13}O^+$	0.41^b ± 0.11	0.42^b ± 0.00	0.63^{ab} ± 0.16	1.05^a ± 0.12	Unidentified	*
137.1	$C_9H_{15}N^+$	0.13 ± 0.19	0.14 ± 0.19	0.29 ± 0.06	0.30 ± 0.04	Pentyl-1H-pyrrole[4]	ns

[1] Instrument database.
[2] Nieva-Echevarría et al. (2017),[31] doi.org/10.1016/j.foodres.2017.06.043.
[3] Mancini et al. (2017),[30] doi.org/10.1017/S1751731117003421.
[4] Nieva-Echevarría et al. (2018),[13] doi.org/10.1016/j.foodres.2017.10.029.
[†] m/z: mass to charge ratio
[‡] ns: not significant (p>0.05); a, b as superscript letters are significant different means at * p<0.05.

Paper 4

Chemical and volatile composition of Atlantic salmon (*Salmo salar* L.) as affected by diet and thermal process

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ABSTRACT

This paper examines the physico-chemical characteristics and volatile organic compounds (VOCs) of Atlantic salmon fillets cooked by boiling and sous-vide (SV). A. salmon were fed diets with two substitution levels of fishmeal by defatted *Hermetia illucens* larvae meal (0 and 100%; H0, H100). Diets did not affect color nor dry matter contents of the fillets. H100 diets increased saturated fatty acid content and thus negatively affected the lipid health indexes. Interestingly, H100 was associated with promising levels of n3 fatty acids, eicosapentaenoic and docosahexaenoic acid. H100 diet led to some modifications in VOC content. Cooking increased the lightness and yellowness indexes, along with secondary oxidation products, without changing the cooking loss and the nutritional value of A. salmon fillets. SV fillets had higher moisture than the boiling ones, whereas boiled fillets had greater VOCs. The 100% substitution level and the cooking by SV or boiling guaranteed good characteristics and nutritional values of the fillets.

KEY-WORDS: *Hermetia illucens*, post-smolt, lipid oxidation, sous-vide cooking.

1. INTRODUCTION

Aquatic animals represent important sources of protein and lipid, especially polyunsaturated fatty acids (PUFA) of the n3 series and their demand has grown exponentially since the '80. To date, aquaculture provides more than 50% of the global seafood consumed and FAO estimated that this percentage will go beyond the 60% in 2030. However, the production of the needed amount of aquacultured species requires high amounts of aquafeeds, thus inducing an overexploitation of natural resources in terms of fish stocks (for fishmeal and fish oil production) and arable land (for leguminous plant production; Boyd & McNevin, 2015). The substitution of dietary fishmeal with insect meal has been proposed as a sustainable and feasible way to raise fish. Nevertheless, diet composition may alter the nutritional quality of fish. The major concern of fishmeal substitution with alternative terrestrial resources, such as insect, is the reduction of PUFA n3 in diet, hence in fish muscle.

The *Hermetia illucens* larvae (H), or black soldier fly, can comprise high lipid and protein concentrations (up to 30% and around 40% of dry weight, respectively), depending on the feeding media (Diener et al., 2009). For example, feeding black soldier fly larvae a diet enriched with fish offal increased the concentration of n3 in the larvae (Sealey et al., 2011; St-Hilaire et al., 2007) and adding seaweeds to the insect substrate can add valuable nutrients to the larvae, such as n3 fatty acid (FA), iodine and vitamin E (Liland et al., 2017).

Additionally, other fish manipulation, such as cooking, may influence lipid composition, because of the thermal processes which lead to lipid oxidation (Luo, Taylor, Nebl, Ng, & Bennett, 2018). Recently the sous-vide (SV) cooking has gained importance. Sous-vide, which literally means ''under vacuum'', is defined as ''foods that are cooked under controlled conditions of temperature and time inside heat stable vacuumed pouches'' (Schellekens, 1996). The common cooking methods, such as boiling (Bo), grilling and baking, are known to deeply impact food flavor, color, texture and nutrients, while the controlled condition of SV seems to carry several advantages. For instance, the vacuum sealing allows an efficient heat transfer from the water to the food and prevents water loss during cooking (Church & Parsons, 2000). In addition, SV may diminish heat damage to protein and lipid as well as to the aromatic compounds (Singh, et al., 2016). In this regard, Nieva-Echevarría et al. (2017) have recently underlined the relevance between the changes provoked by cooking in fish volatile profile and the aromatic compounds. This is because the cooked fish aroma not only depends on the cooking method performed, but also on fish species and fish growing conditions.

Thus, the present work aimed at investigating the effect of *infra vitam* and *post mortem* factors, such as feeding formulation and cooking methods, on the chemical and volatile composition of farmed A. salmon (*Salmo salar*). Partially defatted H meal totally substituted fishmeal in A. Salmon diets, while sous-vide cooking was compared to the boiling practice.

2 MATERIALS AND METHODS

2.1 Fish farming and sampling

Salmon were raised in cages in an open-water system. The fish were divided into two separate cages, corresponding to two experimental groups for the feed formulation. The first diet contained fishmeal, soy protein concentrate, pea protein concentrate, corn gluten meal and wheat gluten meal as protein source was set as control (H0) and the other diet presented a substitution of the 100% (H100) of the fishmeal with partially defatted H meal. The larvae were grown on media partially containing *Ascophyllum nodosum* mixed with organic waste streams (60:40).The diets were formulated to be isonitrogenous (crude protein –about 39 g/100 g dry matter, DM), isolipidic (about 29 g/100 g DM), and isoenergetic, by adjusting fish oil (increasing from about 10% to about 15% of feed composition in H0 and H100, respectively). At the end of the trial, fish were individually weighed (average weight of 3610 ± 366 g) and slaughtered by manual

percussion. Then, they were gutted and filleted. Six fillets (the weight of each was about 1138.10 ± 135.60 g) for each group were brought to the Department of Agriculture, Food, Environmental and Forestry Sciences (DAGRI). The fillets were vacuum packed and frozen at −80 °C until analyses. Further details on the feed production, feeding trial and growth performance with A. salmon were reported elsewhere (Belghit et al., 2019).

2.2 Cooking trial

Six fillets per group were cut into 4 sections (around 7 cm width), corresponding to the cranial, two middle (M1 and M2), and the caudal parts. Each middle section was weighted (M1: 279.60 ± 38.34 g; M2: 250.94 ± 42.41 g) and the thickness was measured (M1: 32.50 ± 4.87 mm; M2: 30.91 ± 4.69 mm) in order to optimize the cooking processes, as follows.

Food thickness affects heating transfer inside the food matrix (Baldwin, 2012), hence changing the time needed for reaching the target temperature at the core. Since the fillets were not uniform, we performed a preliminary trial in order to evaluate the time needed to reach 60 °C at the core of fillets (pasteurization temperature proposed by Baldwin, 2012) with different thickness (from 2.5 to 4 cm) by boiling the fillets in water at 100 °C. Furthermore, we referred to Baldwin (2012) to know the length of sous-vide cooking of fish slides (thickness from 2.5 to 4 cm) in water at 60 °C in order to reach 60 °C at the core. This was necessary since the insertion of a thermometer inside the vacuum bag would surely have altered the cooking conditions. Once the time needed to reach 60 °C at the core of the fillets was over (approximately 15 min for boiled fillets and from 50 to 70 min for sous-vided ones), we decided to maintain the fillets immersed in water for an additional time (<30 s following Baldwin, 2012) necessary to pasteurize the product. M1 and M2 were destined to boiling and sous-vide cooking, respectively, while the other two portions (cranial and caudal regions) were utilized for analyses of the raw fish. M1 was inserted into commercial plastic bags suitable for cooking (Cuki, La Terrasse, France), which were left open during the thermal process, then put into a thermal bath (Nahita Digital Water Bath, Auxilab S.L., Beriáin, Spain) containing water set at at 100 °C. The M2 was put under-vacuum inside polyethylene bags (Reber, Luzzara, Reggio Emilia, Italy), specific for sous-vide cooking, then completely immersed into an SV 2447 vacuum cooker (Severin, Sauerland, Germany) filled with water. Table S1 reports the calculated cooking time adopted for each sample based on its thickness.

2.3 Physical and chemical analyses

2.3.1 Color, cooking loss

Flesh color (L*, a*, and b*; CIE, 1976) was measured on 8 points of the raw fillets (2 for each regions) and on 2 points of the cooked M1 and M2 portions, using a Konica Minolta colorimeter (Chiyoda, Tokyo, Japan). Cooking loss was

calculated as the weight difference before and after cooking divided by the water content before and after cooking.

2.3.2 Proximate composition, total lipid and fatty acid composition

Firstly, samples were analyzed for moisture, crude protein (N×6.25) and ash contents using AOAC official methods (2012). Both raw and cooked fillets underwent lipid extraction (Folch et al. 1957), then the lipid extracts were gravimetrically quantified, trans-esterified and analyzed for FA profile by gas-chromatography (GC) as described by Secci et al. (2018). The analysis was conducted using a Varian GC 430 gas chromatograph (Agilent, Palo Alto, CA, USA), equipped with a flame ionization detector (FID); a Supelco OmegawaxTM 320 capillary column (30 m, 0.32 mm, i.d., 0.25 µm film and polyethylene glycol bonded phase; Supelco, Bellefonte, PA, USA) set as detailed in Secci et al. (2018). Tricosanoic acid (C23:0) (Supelco, Bellefonte, PA, USA) was chosen as internal standard; FAs were identified by comparing the fatty acid methyl ester (FAME) retention time with Supelco 37 component FAME mix (Supelco, Bellefonte, PA, USA). From the FA profile, fat quality indexes as atherogenicity index (AI), thrombogenicity index (TI) and hypocholesterolemic/hypercholesterolemic FA ratio (h/H) were calculated following the equation proposed by Ulbricht and Southgate (1991) and Santos-Silva et al. (2002).

2.3.3 Lipid oxidation: TBARS and VOCs

The 2-thiobarbituric acid reactive substances (TBARS) were measured according to Vyncke (1970). Results were expressed as mg of malondialdehyde (MDA) equivalents/kg sample. A commercial proton transfer reaction-time of flight-mass spectrometer (PTR-ToF-MS) 8000 model (Ionicon Analytik GmbH, Innsbruck, Austria) in its standard configuration was used for VOC measurements of feeds and fillets, using H3O+ for the proton-transfer reaction (see (Ellis and Mayhew, 2014) for a detailed description of the technology). All mass spectra have been sampled ranging between m/z = 15 and m/z = 250. VOC measurements were performed using the system proposed by (Colzi et al., 2017). Briefly, each fillet sample was put in a 3/4 L glass jar and topped with a special glass cap that allows the connection between the inlet of the PTR-ToF-MS, the headspace collecting and the zero-air generator. Sample temperatures were 18 °C for raw one and 36 °C of cooked ones. To avoid possible systematic errors, the apparatus was flushed with clean air for 1 min between the measurement of a sample to the next one. The signal intensity of each sample was recorded for 100 s, allowing the acquisition of 100 spectra (1spectrum/1sec). The spectra calibration was performed offline, using the following compounds: $H_3{}^{18}O^+$ (water isotope, detected at m/z = 21.022); NO^+ (nitric oxide, detected at m/z = 29.999) and $C_3H_7O^+$ (acetone, detected at m/z = 59.049). To acquire the raw data (number of counts per second, cps), TofDaq software (Tofwerk AG, Switzerland) was used. Raw data were corrected to ppb according to the formula described by (Lindinger and Jordan, 1998), using a constant value for the reaction rate coefficient (kR = 2×10^{-9} cm^3/s). Afterwards, following the procedure described

by (Aprea et al., 2015), data were filtered by eliminating peaks imputable to water chemistry and to interfering ions, and also all peaks whose average concentrations were lower than a threshold of 0.25 ppb, determined on empirical basis. This filtering enabled to discard signals pertaining ions present in trace amounts. Finally, the tentative identification of VOCs was obtained comparing the measured masses with those reported in the database of the instrument and in the literature (Mancini et al., 2017; Nieva-Echevarría et al., 2018).

2.4 Statistical analysis

A two-way ANOVA analysis was applied with the diet (D; two levels: Ho and H100) and the cooking treatment (T; three levels: raw, Bo and SV) as fixed factors. Interaction D × T was assessed. The significance level was set at 5% and the differences were assessed using Tukey's test. Variability was expressed as Root Mean Square Error (RMSE).

3. RESULTS AND DISCUSSION

3.1 Color and cooking loss

The color of the flesh is an important criterion for the quality of raw salmon and hence crucial for marketing. Moreover, many consumers consider the color of cooked meat as a reliable indicator for doneness and safety (Pathare & Roskilly, 2016). The diet did not impart any effect on the color of A. salmon fillets (Table 1), in line with Renna et al. (2017). Considering the cooking treatment, heated flesh is characterized by higher L* and b* indexes than raw samples regardless of the administered diet (Table 1), probably because of the carotenoid oxidation, of the Maillard reactions, of the degradation of lipids and of the protein-lipid browning (Haard, 1992). However, L* and b* between Bo and SV did not significantly differ ($p>0.05$), probably because the same core temperature was reached and held for the same time (15 minutes). The a* index was the same in raw and cooked fillets. A. salmon is well-known for its pink-red color and a more intense red color is perceived as higher freshness, better flavor and higher quality. In this regard, the lack of a significant difference between the cooking treatments enables to suppose that both Bo and SV treatments at the condition tested maintained the original color of fillets.

Cooking loss is a critical factor in the meat industry as it determines the technological yield of the cooking process and generally mirrors the degree of protein denaturation. Neither the diet nor the treatment significantly influenced the cooking loss of the fillets, whose calculated values were 13.63 and 12.19% in Ho and H100 groups, respectively, and 14.06 and 11.77%, in Bo and SV cooked fillets, respectively. The present findings were in line with those of Secci et al. (2019), who stated that conventional protein source substitution had no effect on the cooking yield of fillets of rainbow trout (*Oncorhynchus mykiss*) fed diets including H meal. The temperature, the cooking method and the cooking time are factors guiding cooking loss (Kumar & Aalbersberg, 2006). According to del Pulgar

et al. (2012), cooking losses were lower in samples cooked for a shorter time and at a lower temperature ($p<0.001$). Kato et al. (2016) found that the SV prepared at 65 °C for 12.5 minutes showed the best results in terms of cooking loss. Therefore, our outcomes were coherent with the previous results, and the cooking loss of the two cooking methods were equal since the temperature × time combination at the samples' center were unified.

3.2 Proximate composition, total lipid and fatty acid composition

The proximate composition of A. salmon fillets is reported in Table 2. The use of H100 (inclusion level of 150 g kg^{-1} diet) did not affect the proximate composition, except for the moisture, which decreased in H100. Analogously, Iaconisi et al. (2017) found that the water content of blackspot sea bream (*Pagellus bogaraveo*) fillets decreased with the increasing levels of *Tenebrio molitor* meal in feed (tested up to a 400 g kg^{-1} inclusion). Furthermore, even the dry matter content of fillets from rainbow trout fed with diets containing 200 and 400 g kg^{-1} of H, increased gradually compared to diets devoid of H (Renna et al., 2017). In the present work, taking the cooking methods into account, moisture and protein contents were significantly lower in Bo and SV samples compared to raw ones, while the lipid and ash contents were not affected (Table 2). Remarkably, the SV fillets had a slightly higher water content ($p=0.056$) than Bo; an explanation is that the SV packaging probably prevents the water loss, according to Church and Parsons (2000). Nonetheless, the Bo and SV fillets exhibited the same significant increase in protein content compared to the raw fillets, coherently with Türkkan et al. (2008), who found significant changes in protein contents for all the conventional methods tested.

The FA profile is reported in Table 3. As previously found for rainbow trout (Secci et al., 2019), the H100 significantly changed fish overall FA profile. Specifically, saturated FA (SFA) significantly increased to the detriment of monounsaturated FA (MUFA) and PUFA n6. A considerable rise of PUFA n3 was also noted the A. salmon fed H100, compared to the Ho group. These findings were consistent with the fact that the FA composition of muscle is to a great extent correlated with that of the dietary composition (Li et al., 2016). Especially, this emerged looking at the presence of SFA, particularly lauric acid (C12:0), which are distinctive of *H. illucens* (Barroso et al., 2017). Since *H. illucens* has an abundant lipid content, the general strategy to obtain isoenergetic and isolipidic diets containing H meal is to reduce the amount of fish oil. This generally leads to a dramatic decrease in PUFAn3. For this reason, the H100 diet of the present study was produced with around 15% of fish oil (10% in Ho diet). Another crucial factor controlling the n3 content in the final product is largely dependent on the rearing substrate of insects (St. Hilaire et al., 2007; Barroso et al., 2014). Accordingly, feeding insects with a diet enriched with *A. nodosum*, macroalgae that are known to contain long chain n3 PUFA, brought about a significant higher percentage of PUFA, particularly EPA levels in the *H. illucens* larvae (Liland et al., 2017).

Because of the FA modification due to the diet, the nutritional indexes IA, IT, h/H and the overall EPA+DHA content were examined and are displayed in Table 3. Apart from h/H, the indexes showed to be significantly higher in H100 than in Ho group, likely because of the high SFA content of the H100 diet. These results were in line with Renna et al. (2017) and Iaconisi et al. (2017), who declared that the partial fishmeal substitution with defatted H meal and with full-fat *T. molitor* meal, respectively, had adverse effect on AI and TI. Nevertheless, the AI and TI values of the present study could be considered healthy for consumers as they were less than 1.0 (Łuczyńska et al. 2017; Renna e al., 2017). EPA and DHA are essential FA for fish and humans that play an important role in reducing the risk of cardiovascular and inflammatory human diseases (Siriwardhana et al., 2012). Interestingly, observing Table 3, it is reasonable to assert that the feeding strategies adopted here (algae in insect's rearing substrates, dietary fish oil increase) were able to contrast the negative effect of H meal on fish FA composition, guaranteeing salmon nutritional quality.

Even the cooking methods did not alter the nutritional profile of the raw fish, in line with the results reported by Bastías et al. (2017). Our results confirmed that by controlling temperature and cooking time, efficient results in terms of a reduced change in the FA content of the product can be achieved. The same observation was obtained by Garcia-Linares et al. (2004), who pointed out that salmon FA profile did not differ during SV treatment. Because of unmodified FA fraction, the nutritional indices of Bo and SV fillets did not differ (Table 3). Among the other, EPA+DHA content is a crucial index, since FAO/WHO recommends a daily intake of at least 500 mg/d EPA+DHA for adults for the prevention of cardiovascular disease. According to Jensen et al. (2012), 100 g of farmed A. salmon fillet provided about 1 g of EPA and DHA. In this study, 100 g of cooked A. salmon fillet contained an average of 1.5 g of EPA and DHA, irrespective of the methods applied, and this quantity is three times higher than the minimum daily recommended dose. Accordingly, the suggested weekly amount of EPA and DHA could be reached by consuming 230 g of cooked A. salmon.

3.3 Lipid Oxidation: TBARS and VOCs

The content of secondary oxidation products is reported in Figure 1. TBARS were not influenced by the diet. Our findings closely resembled a study carried out by Altmann et al. (2018) who pointed out negligible effects of the dietary inclusion of a microalga or H meal on the TBARS content in broiler meat. Considering the treatments, the cooking led to a significant increase in TBARS content. This result agreed with those reported by Ramos et al. (2016) for Tambaqui fillets (*Colossoma macropomum*), stating that raw and SV fillets differed significantly after processing.

An equal level of TBARS in Bo and SV samples was found; theoretically, since SV treatments are characterized by the absence of oxygen, SV had been supposed to show lower values in comparison with Bo. In our case, the decreased

TBARS content in Bo samples could depend on the decomposition of secondary oxidation products into volatile molecules, as confirmed by Table 4.

The total replacement of dietary fishmeal with H meal led to significant changes in VOCs (Table 4), such as an increase in the content of formaldehyde, propanal and heptadienal, while it resulted in lower acetic acid content in H100 compared to Ho. Previous findings indicated significant changes in the perceived intensity of aroma and flavor in rainbow trout as a function of substituting fishmeal with different percentage of H meal in the diet composition (Borgogno et al., 2017). Additionally, Mancini et al. (2017) stated that only the heptanal and octanal concentrations were affected in rainbow trout fillets fed with 25% and 50% substitutions of fishmeal with H meal.

Considering the cooking treatments, even if not all the volatile carbonyl molecule compounds were influenced (Table 4), a general trend discerned that the compounds exhibited significant differences, such as acetylene, formaldehyde, acetaldehyde, propanal, heptadienal, showing the lowest values for raw samples and the highest for Bo samples (with the SV samples in between Bo and raw). These differences might be reasonable since raw flesh has few aromas and the flavor of cooked meat is developed during cooking (del Pulgar et al., 2013). The thermal process induces Maillard reaction of amino acid or peptides, reducing sugars such as 5-methylfurfural (Morita et al., 2003), which can explain the highest VOCs content in Bo samples. The aldehydes were the predominant VOCs in cooked A. salmon, aligning with results on obscure puffer (*Takifugu obscurus*) (Tao et al. (2014). Nieva-Echevarría (2017) recently studied how acetaldehyde, acetylene and formaldehyde are responsible for the aromatic profile of European sea bass fillets (*Dicentrarchus labrax*) defined as "pungent", while Li et al. (2018) verified that the heat processing affected the VOCs of vacuum-packed silver carp (*Hypophthalmichthys molitrix*) by forming mainly aldehydes and alcohols. It is commonly accepted that the aroma and flavor of cooked flesh play a key-role in meat quality, acceptance and preference by consumers. For this reason, sensory assessments should be performed to better understand if people are able to discriminate among different cooking methods.

4. CONCLUSIONS

In conclusion, the entire substitution of fishmeal with partially defatted H meal could be possible in farmed A. salmon, without worries about diminishing PUFA n3 in fish fillets. In addition, rearing insects on algae and including fish oil in the diet containing insect safeguarded the nutritional value of A. salmon. Moreover, the main findings indicated an alteration in some FA composition and VOCs of the fillets. Nevertheless, the outcome had satisfactory physical and chemical quality. Furthermore, the results of this work highlighted that the SV and Bo fish fillets have almost similar effect on chemical, physical and nutritional compositions of fish fillets when the temperature and time were controlled. Yet, SV fillets had priority over the Bo ones regarding the shelf life whilst the Bo fillets were marked

by higher VOC content. Further studies focusing on the cost and economic feasibility of using algae as insect rearing substrate are encouraged.

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Table 1. Color parameters values of A. salmon fillets fed diets with two inclusion levels of defatted meal of *H. illucens* (H0=0% *H. illucens*; H100=100% *H. illucens*) analyzed as raw and subjected to boiling (Bo) and sous-vide (SV) cooking methods.

	Diet (D)		Treatment (T)			p-value[1]			RMSE[2]
	Ho	**H100**	**Raw**	**Bo**	**SV**	**D**	**T**	**D×T**	
L*	66.22	66.17	45.53^{y}	76.41^{x}	76.64^{x}	*NS*	***	*NS*	3.407
a*	11.16	11.67	11.24	11.80	11.20	*NS*	*NS*	*NS*	2.650
b*	15.68	15.81	11.77^{y}	18.98^{x}	16.49^{x}	*NS*	***	*NS*	3.170

[1] NS: not significant; ***p<0.001. x, y as superscript letters are significant different means between cooking treatments at (p<0.05).
[2] RMSE: Root Mean Square Error.

Table 2. Proximate composition (g/100 g) of A. salmon fillets fed diets with two inclusion levels of *H. illucens* defatted meal (H=0% *H. illucens*; H100=100% *H. illucens*) analyzed as raw and subjected boiling (Bo) and sous-vide (SV) cooking methods.

	Diet (D)		Treatment (T)			p-value[1]			RMSE[2]
	Ho	**H100**	**Raw**	**Bo**	**SV**	**D**	**T**	**D×T**	
Moisture	63.24^{a}	61.09^{b}	64.27^{x}	60.13^{y}	62.11^{xy}	*	**	NS	2.66
Protein	20.70	21.41	19.05^{y}	22.34^{x}	21.77^{x}	NS	***	NS	1.66
Ash	1.38	1.45	1.32	1.48	1.46	NS	NS	NS	0.21
Lipid	13.28	13.98	13.70	13.95	13.24	NS	NS	NS	2.05

[1]NS: not significant; * p<0.05; ** p<0.01; *** p<0.001. a, b as superscript letters are significant different means between diets (p<0.05); x, y as superscript letters are significant different means between cooking treatments at (p<0.05).
[2]RMSE: Root Mean Square Error.

Table 3. Fatty acid profile (g/100 g of total fatty acids), nutritional index (AI, TI, h/H) and EPA+DHA content (g/100 g of fillet) of A. salmon fillets fed diets with two inclusion levels of *H. illucens* defatted meal (H0=0% *H. illucens*; H100=100% *H. illucens*) analyzed as raw and subjected to boiling (Bo) and sous-vide (SV) cooking methods.

	Diet (D)		Treatment (T)			p-value[1]			RMSE[2]
	H0	H100	Raw	Bo	SV	D	T	D×T	
C12:0	0.122b	1.798a	0.929	0.993	0.960	***	NS	NS	0.146
C14:0	1.903^{b}	2.776^{a}	2.317	2.361	2.341	***	NS	NS	0.100
C16:0	8.376^{b}	8.980^{a}	0.174	0.173	0.173	***	NS	NS	0.160
C16:1n7	1.790^{b}	2.260^{a}	0.293	0.301	0.300	***	NS	NS	0.065
C18:0	2.216^{b}	2.304^{a}	2.292	2.455	2.242	**	NS	NS	0.081
C18:1n9	34.627^{a}	29.602^{b}	32.180	32.002	32.162	***	NS	NS	0.558
C18:1n7	2.364^{a}	2.190^{b}	2.275	2.276	2.280	***	NS	NS	0.029
C18:2n6	12.504^{a}	11.085^{b}	11.792	11.796	11.796	***	NS	NS	0.252
C18:3n3	5.113^{a}	4.385^{b}	4.736	4.762	4.748	***	NS	NS	0.195
C20:1n9	5.034^{a}	5.647^{b}	5.330	5.360	5.332	***	NS	NS	0.136
C20:2n6	1.240^{a}	1.075^{b}	1.159	1.158	1.155	***	NS	NS	0.039
C20:4n3	1.083^{b}	1.159^{a}	1.117	1.135	1.111	*	NS	NS	0.095
C20:5n3	3.558^{b}	3.981^{a}	3.772	3.796	3.740	***	NS	NS	0.200
C22:1n11	4.188^{b}	5.431^{a}	4.792	4.841	4.796	***	NS	NS	0.142
C22:5n3	1.833^{b}	2.280^{a}	2.050	2.080	2.039	***	NS	NS	0.153
C22:6n3	8.357^{b}	8.929^{a}	8.632	8.641	8.656	*	NS	NS	0.685
ΣSFA	13.534^{b}	16.794^{a}	15.173	15.172	15.146	***	NS	NS	0.383
ΣMUFA	49.280^{a}	46.505^{b}	47.917	47.840	47.921	***	NS	NS	0.545
ΣPUFAn6	14.744^{a}	13.045^{b}	13.894	13.870	13.919	***	NS	NS	0.183
ΣPUFAn3	21.557^{b}	22.382^{a}	21.945	22.032	21.931	**	NS	NS	0.705
AI[3]	0.19^{b}	0.27^{a}	0.23	0.23	0.23	***	NS	NS	0.01
TI[4]	0.11^{b}	0.13^{a}	0.12	0.12	0.12	***	NS	NS	0.01
h/H[5]	5.7^{a}	4.46^{b}	5.07	5.06	5.07	***	NS	NS	0.16
EPA+DHA	1.37	1.59	1.49	1.52	1.43	***	NS	NS	0.169

[1] NS: not significant; * p<0.05; ** p<0.01; *** p<0.001. a, b as superscript letters are significant different means between diets (p<0.05).

[2] RMSE: Root Mean Square Error.

Σ were obtained by summing even the fatty acids contained at level <1 g/100 g total fatty acids as C13:0, iso-C14:0, C14:1 n5, iso-C15:0, C15:0, iso-C16:0, C16:1n9, C17:0, C17:1, C16:4n1, C18:3n6, C18:4n3, C14:4n1, C20:0, C20:1n11, C20:1n7,C20:3n6, C20:4n6, C20:3n3, C22:0, C22:1n9, C22:1n7, C22:2n6, C21:5n3, C22:4n6, C22:5n6, C24:0.

[3] AI: atherogenicity index

[4] TI: thrombogenicity index

[5] h/H: hypocholesterolaemic/hypercholesterolaemic fatty acid ratio

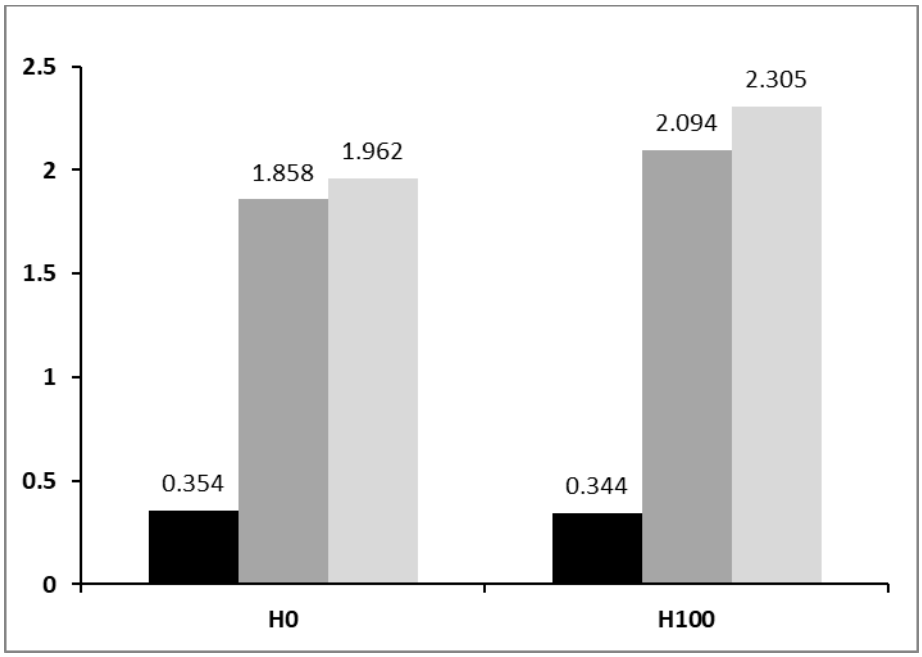
Table 4. Volatile organic compounds (ppb) in A. salmon fillets fed diets with two inclusion levels of H. illucens defatted meal (Ho=0% H. illucens; H100=100% H. illucens) analyzed as raw and subjected to boiling (Bo) and sous-vide (SV) cooking methods.

Components	Identification	Diet (D)		Treatment (T)			p-value[4]			RMSE[5]
		Ho	H100	Raw	Bo	SV	D	T	D×T	
CH_3+	*Unidentified*	20.333	22.415	8.851^{z}	24.011^{x}	18.736^{y}	NS	*	NS	4.753
C_2H_3+	Acetylene[1]	33.320	41.040	9.179^{z}	45.407^{x}	28.953^{y}	NS	**	NS	11.921
CH_3O+	Formaldehyde	71.252^{b}	127.170^{a}	6.710^{z}	137.727^{x}	60.695^{y}	**	***	NS	32.138
CH_4O+	Methanol	13.910	13.577	6.634	14.510	12.977	NS	NS	NS	3.438
C_3H_5+	*Unidentified*	36.094^{b}	57.626^{a}	6.197^{z}	59.320^{x}	34.400^{y}	**	**	NS	14.883
C_2H_3O+	*Unidentified*	26.953	30.992	11.731	31.090	26.856	NS	NS	NS	8.477
C_3H_7+	*Unidentified*	6.447	7.249	3.092	7.476	6.220	NS	NS	NS	2.085
C_2H_5O+	Acetaldehyde	446.057	560.465	54.142^{z}	637.651^{x}	368.871^{y}	NS	**	NS	158.005
C_2H_7O+	Etanol	1.494	1.594	0.273^{z}	1.752^{x}	1.337^{y}	NS	*	NS	0.417
CH_5S+	Methanethiol[2]	1.314	2.428	0.000^{y}	3.248^{x}	0.494^{y}	NS	**	NS	1.471
C_4H_7+	*Unidentified*	10.252^{b}	15.395^{a}	2.417^{z}	16.265^{x}	9.381^{y}	*	**	NS	4.646
C_3H_5O+	*Unidentified*	1.888^{b}	3.088^{a}	0.366^{z}	3.252^{x}	1.724^{y}	**	**	*	0.816
C_4H_9+	*Unidentified*	1.602	2.067	1.640	2.174	1.495	NS	NS	NS	0.758
C_3H_7O+	Propanal[3]	59.318^{b}	92.302^{a}	16.089^{z}	99.398^{x}	52.222^{y}	**	***	NS	18.289
$C_2H_5O_2$+	Acetic acid	0.989^{a}	0.203^{b}	1.834^{x}	0.000^{y}	1.192^{xy}	*	**	*	0.751
C_2H_7S+	Ethanthiol[2]	0.190	0.327	0.057	0.323	0.195	NS	NS	NS	0.174
C_5H_7+	*Unidentified*	0.850^{b}	1.217^{a}	0.562	1.185	0.882	*	NS	NS	0.359
$C_5H_9C_2H_{13}S$+	*Unidentified*	4.902	6.624	0.748	6.103	5.423	NS	NS	NS	4.002
C_4H_7O+	*Unidentified*	2.087	1.645	0.025	1.438	2.294	NS	NS	NS	1.169
C_4H_9O+	Isobutanale	2.963	3.583	0.590^{z}	3.749^{x}	2.796^{y}	NS	*	NS	0.927
C_3H_7S+	*Unidentified*	18.882	11.947	17.287	14.162	16.668	NS	NS	NS	13.370
$C_2H_5OSC_2H_5O_3$+	*Unidentified*	3.482	2.113	2.433	2.404	3.191	NS	NS	NS	2.295
$C_3H_{11}SC_6H_7$+	*Unidentified*	0.486	0.503	0.377	0.510	0.479	NS	NS	NS	0.122
C_2H_7N+	Methylpyrrol	1.092	1.218	0.404	1.299	1.011	NS	NS	NS	0.330
C_6H_{11}+	*Unidentified*	2.085	3.002	0.274	3.079	2.008	NS	NS	NS	1.318
$C_5H_{11}O$+	*Unidentified*	0.435	0.622	0.000^{z}	0.633^{x}	0.424^{y}	NS	*	NS	0.166
$C_4H_9O_2$+	Butirric Acid	0.777	0.419	0.000	0.340	0.856	NS	NS	NS	0.549
C_7H_7+	*Unidentified*	0.309	0.232	0.743	0.294	0.247	NS	NS	NS	0.263
C_7H_9+	*Unidentified*	0.744	0.581	0.753	0.512	0.812	NS	NS	NS	0.492
$C_2H_7O_2S$+	Methanthiol	0.354	0.224	0.146	0.281	0.298	NS	NS	NS	0.275
C_7H_{11}+	*Unidentified*	0.231	0.360	0.026	0.343	0.248	NS	NS	NS	0.167

C_7H_{13}+	*Unidentified*	0.357	0.445	0.058^{z}	0.494^{x}	0.308^{y}	NS	*	NS	0.187
C_8H_9+	*Unidentified*	0.231	0.135	0.496	0.110	0.256	NS	NS	NS	0.209
C_7H_6O+	Benzaldeide[2]	0.727	0.534	0.851	0.606	0.655	NS	NS	NS	0.439
C_8H_{13}+	*Unidentified*	0.433	0.529	0.409	0.524	0.438	NS	NS	NS	0.152
$C_7H_{11}O$+	Heptadienal	0.000^{b}	0.124^{a}	0.000^{y}	0.124^{x}	0.000^{y}	**	**	**	0.072
C_9H_{13}+	*Unidentified*	0.412	0.190	0.301	0.708	0.301	NS	NS	NS	0.367
$C_2H_{13}O_7$+	*Unidentified*	0.211	0.074	0.137	0.105	0.180	NS	NS	NS	0.220

[1] Instrument database
[2] Nieva-Echevarría et al., 2017.
[3] Mancini et al., 2017.
[4] NS: not significant; * p<0.05; ** p<0.01; *** p<0.001. a, b as superscript letters are significant different means between diets (p<0.05); x, y, z as superscript letters are significant different means between cooking treatments at (p<0.05).
[5] RMSE: Root Mean Square Error.

Figure 1. Graph bars of TBARS levels (mg MDA-eq/kg fillet) of A. salmon fillets fed diets with two inclusion levels of *H. illucens* defatted meal (H0=0% *H. illucens*; H100=100% *H. illucens*) analyzed as raw (black bars) and subjected to boiling (Bo, dark grey bars) and sous-vide (SV, light grey bars) cooking methods.



x, y: are significant different mean among treatments ($p<0.05$).
Diet and Diet x Treatment were not found significantly different ($p>0.05$).
RMSE: Root Mean Square Error equal to 0.573.

Table S1. Cooking time utilized for each fillet.

Sample n.	Time		
	Thickness (cm)	Boiling	Sous-vide
1 Ho	2.5	16′ & 30″	52′
2 Ho	3.5	17′ & 30″	75′
3 Ho	4	17′ & 80″	75′
4 Ho	3	17′	62′
5 Ho	2.5	16′ & 30″	52′
6 Ho	3	17	62′
1 H100	3.5	17′ & 30″	75′
2 H100	3	17	62′
3 H100	3.5	17′ & 30″	75′
4 H100	3	17	52′
5 H100	3.5	17′ & 30″	75′
6 H100	3	17	62′

Paper 5

Full title: A commercial macroalgae extract in a plant-protein rich diet diminished saturated fatty acids of *Oncorhynchus mykiss* Walbaum fillets

Short title: Macroalgae extract in diets affects rainbow trout fillet quality

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Abstract

Background: Seaweeds are considered both novel feed ingredients, nutraceutical compounds and source of pigments and protein. They appear to possess bioactive properties, such as hypolipidemic, antioxidant and immune-stimulative actions; furthermore, their proteins are considered just as nutritious as terrestrial vegetables.

In the present work, rainbow trout (*Oncorhynchus mykiss* Walbaum) was fed for 95 days with three diets: a fishmeal-based positive control diet (C+), a vegetable protein-based negative control diet (C-) and a diet similar to C- where 5% of soybean concentrate was replaced by a commercial blend of seaweeds. The monitored parameters were fish performances, physical and marketable characteristics, fillet oxidative status and fatty acid content. The estimated indices of enzyme activities involved in lipid metabolism were calculated.

Results: Fish performances were reduced in C- and T fish; C- and T fillet fatty acid (FA) profile globally showed the same pattern and was distinct from C+, i.e., lower content of polyunsaturated FAs and higher content of monounsaturated and polyunsaturated FAs n6; contrarywise, saturated FAs were significantly lower in T group in comparison to the other two groups ($P<0.01$). The estimated indices of enzyme activity highlighted differences between dietary groups; desaturase activities of C18 and n3 FAs were higher in T in comparison to C- ($P<0.001$). A

slight impoverishment of antioxidant activity was found in T compared to C+ group.

Conclusion: Dietary seaweeds seemed to be capable of influencing fillet FA composition as well as the activity of enzymes related to lipid metabolism. Further studies in this regard are encouraged.

Keywords: Algae; seaweed; rainbow trout; saturated fatty acid; quality; *Oncorhynchus mykiss*.

1. Introduction

The intensification and expansion of farmed fish production necessitates larger volumes of protein for feed formulation and in the last decade vegetable proteins have been the chief substitute of fishmeal. Aquaculture industry takes particular consideration for this aspect as vegetable substitutes rarely have an optimal fatty acid (FA) profile and often contain antinutritive factors (Oliva-Teles, Enes, & Peres, 2015). Indeed, fish products are well-known for their high polyunsaturated fatty acid (PUFA) content and feed FA profile could negatively affect the fish characteristics. As counter-action, supplements and functional ingredients could be added to feeds in order to carry nutraceutical molecules and have been studied to leap over the hurdles carried by vegetable sources.

Among functional ingredients, seaweeds are promising. Base of the aquatic food chain (Norambuena et al., 2015) and thus eco-friendly, with a potentially high availability, their production lined up at 30.1 million tonnes (wet weight) in 2016 (FAO, 2018). For a long time, macroalgae have been used for ecosystem services, in polyculture systems and in water remediation; more recently, research has pointed in the direction of using them as fodder supplements and alternative protein source for farmed fish (Hasan & Chakrabarti, 2009). Indeed, macroalgae enclose in themselves an enormous potential, being a source of polysaccharides, lipid, protein, minerals, vitamins, various pigments and polyphenols (Holdt & Kraan, 2011). The nutritional value of algal proteins is no less than that of terrestrial vegetables. Macroalgae typically contain a low lipid amount, but the predominant FAs are eicosapentaenoic acid (EPA) and other n3 and n6 PUFA, in a balanced mix (Holdt & Kraan, 2011). In addition, algae contain compounds with bioactive features, such as immune-stimulative properties, antibacterial and antiviral actions, hypocholesterolaemic and hypolipidemic effects and antioxidant activity (Holdt & Kraan, 2011; Samarakoon & Jeon, 2012). However, caution must be kept as regards too high concentrations of minerals or the presence of heavy metals; moreover, the chemical composition is subjected to high variation depending on species, environment, time and geographic space, therefore generalisations about their characteristics should not be made (Hasan & Chakrabarti, 2009; Holdt & Kraan, 2011). There are 33,260 species named in AlgaeBase (Guiry & Guiry, 2017), usually grouped for commercial purposes in green, red, brown, yellow-green algae, roughly corresponding to Chlorophyta,

Rhodophyta, Phaeophyceae and Xanthophyceae taxonomic groups, but many other groups exist (Hasan & Chakrabarti, 2009).

As a consequence of such a variety, different results can be found in the literature. For instance, some research studies did not find any effect of a low-level inclusion of dietary seaweed on growth performances and protein efficiency ratio of several fish species (Güroy, Ergün, Merrifield, & Güroy, 2013; Hasan & Chakrabarti, 2009; Peixoto et al., 2016; Soler-Vila, Coughlan, Guiry, & Kraan, 2009). However, early studies have shown that low levels (2.5–10% of the diet) of algae in fish diets have positive effects on many characteristics of fish, such as growth, disease resistance and end-product quality, while other studies detected negative results (Norambuena et al., 2015).

The present work aimed to assess the suitability of replacing 5% of soybean concentrate with a commercial blend of seaweeds in a feed for rainbow trout (*Oncorhynchus mykiss* Walbaum) with a high content of vegetable sources, monitoring its effects on growth performances, fillet marketable, physical and chemical composition.

2. Material and methods

2.1. Experimental diets and growth trial

The present trial was performed according to the European Directive 2010/63/EU (Directive 2010/63/EU, 2010) of the European Parliament and of the Council of European Union on the protection of animals used for scientific purposes. The fish growth and sampling phases were performed at the Edmund Mach Foundation experimental fish plant, San Michele all'Adige, Trento, Italy (Ministerial Clearance n. 120/2008-A).

A number of 900 female rainbow trout (183.3 ± 29.6 g, mean ± SD) was randomly divided into three dietary groups and allocated to a total of nine fiberglass tanks (three tanks per dietary group). The water volume in each tank was set to 3.5 m^3, in a flow-through system (mean flow 4.85 L/sec; mean temperature 12.1°C). Water temperature and dissolved oxygen in the water outlet was measured weekly, while the administered feed and the mortality was recorded daily for each tank. Fish were fed manually six days a week. Feeding ratios were adjusted weekly as the fish grew (total biomass was assessed every third week). Feed conversion ratio (FCR), specific growth rate (SGR) and protein efficiency ratio (PER) were evaluated at the end of trial.

After 2 weeks of acclimatisation, fish were fed for 95 days (65 meals) with three different isonitrogenous, isolipidic and isoenergetic diets (Table 1). A positive control (C+) was formulated containing 53% of fish meal and 15.1% of fish oil (w/w); a negative control (C-) was represented by a diet where marine sources were strongly substituted by protein and oil from vegetable sources; the third diet (T) was formulated basing on C- with a 5% replacement of the soybean meal with a commercial blend of seaweeds. The colour and the FA profile of the

three experimental diets were analysed with the methodology hereunder described.

2.2. Sampling and marketable traits

Three sampling times were set. Six fish in total were sampled after the acclimatisation (To), six fish per dietary group were sampled after six weeks (T1) from the beginning of the experimental feeding and at the end of the trial (T2), i.e. when rainbow trout reached the marketable weight and size. Prior to each sampling, the fish were starved for one day, euthanised by a sharp blow to the head, then frozen at -80°C and shipped to the laboratory where fish were stored at the same temperature until the analyses. Prior to analysing, the fish were thawed overnight at +1°C. Firstly, the colour of the skin was measured on triplicate positions (cranial, medial and caudal) on both skin sides with a CHROMA METER CR-200 (Konica Minolta, Singapore Japan) following the CIELab system (CIE, 1976) and recording L* (lightness), a* (redness index) and b* (yellowness index) parameters. Then, the fish were individually weighed, measured, and dissected to calculate the following parameters:

Condition factor, CF (%) $= \frac{BW}{TL^3} \times 100$,

Fillet yield, FY (%) $= \frac{FW}{BW} \times 100$,

Where: BW is the body weight (g), TL is total length (cm), FW is the weight of fillets with skin (g).

The hepatosomatic (HSI) and visceral somatic (VSI) indices were also calculated.

Colour values of both right and left fillets were recorded and ΔE between samples was calculated according to the following formula: $\Delta E_{(\beta-\alpha)} = \left[\left(L^*_\beta - L^*_\alpha\right)^2 + \left(a^*_\beta - a^*_\alpha\right)^2 + \left(b^*_\beta - b^*_\alpha\right)^2\right]^{0.5}$, where α and β represents alternatively the mean colour values of C+, C- and T.

Finally, C+, C- and T right fillets of trout fed the diets for 6 and 12 weeks (T1 and T2, respectively) were analysed as fresh for physical and chemical characteristics.

2.3. Fillet physical characteristics

The values of pH, maximum shear force and water holding capacity (WHC) parameters were considered. The pH value was measured on triplicate fillet positions (cranial, medial and caudal) by a pH-meter SevenGo SG2™ (Mettler-Toledo, Schwerzenbach, Switzerland). Texture was assessed as the maximum shear force value obtained after a 50% Warner-Bratzler shear test; a ZwickRoell® 109 texturometer (Zwick Roell, Ulm, Germany), equipped with a 1 kN load cell

and a straight blade (width of 7 cm), was used to perform the test set at a crosshead speed of 30 mm/min. Afterwards, fillets were skinned, homogenised and used to determine WHC (Iaconisi, Bonelli, Pupino, Gai, & Parisi, 2018) and chemical composition, as described below.

2.4. Fillet chemical characteristics and estimation of indices of elongase and desaturase activity

Proximate composition, total lipid and cholesterol contents as well as fatty acid profile were assessed by the method described by AOAC (AOAC, 2012), Folch *et al*. (Folch, Lees, & Stanley, 1957) and Secci *et al*. (Secci et al., 2018), respectively. To estimate the activities of the enzymes involved in elongation and desaturation of FAs, the ratio of the product to the precursor was calculated (Mattioli et al., 2018). The following equations were used:

$$Thioesterase = C16/C14$$

$$Elongase = C18/C16$$

$$\Delta 9\ desaturase\ (16) = \frac{C16{:}\,1}{C16{:}\,1 + C16} \times 100$$

$$\Delta 9\ desaturase\ (18) = \frac{C18{:}\,1}{C18{:}\,1 + C18} \times 100$$

$$\Delta 9\ desaturase\ (16 + 18) = \frac{C16{:}\,1 + C18{:}\,1}{C16{:}\,1 + C16 + C18{:}\,1 + C18} \times 100$$

$$\Delta 5 + \Delta 6\ desaturase\ (n6) = \frac{C20{:}\,2n6 + C20{:}\,4n6}{C18{:}\,2n6 + C20{:}\,2n6 + C20{:}\,4n6} \times 100$$

$$\Delta 5 + \Delta 6\ desaturase\ (n3) = \frac{C20{:}\,5n3 + C22{:}\,5n3 + C22{:}\,6n3}{C18{:}\,3n3 + C20{:}\,5n3 + C22{:}\,5n3 + C22{:}\,6n3} \times 100$$

2.5. Fillet oxidative status

Antioxidant properties, namely ABTS, DPPH, FRAP, and the secondary lipid oxidation products (thiobarbituric acid reactive substances, TBARS) were evaluated on 2 g of homogenised samples according to Mancini *et al*. (2015) and Vyncke (1970), respectively.

2.6. Statistical analysis

Data of fish at T1 and T2 were analysed with the SAS statistical software (SAS, 2007), by a one-way ANOVA followed by a *post hoc* test.

3. Results

3.1. Characterisation of the experimental diets

The colour values and detailed FA profile of the experimental diets are depicted in Table S1. Summarising the obtained results, the colour indices seemed to differ between the three experimental diets, specifically, L*, a* and b* were lower in C+ in comparison to C- and T; the yellowness index (b*) showed the highest differences between the treatments. The fatty acid profile seemed to be similar between C- and T diets, but deeply different between C+ and the other two diets; SFA and PUFAn6 were lower, while PUFAn3 were higher in C+ in comparison to C- and T (Table 1), hence, the n3/n6 ratio was higher in C+ diet in comparison to C- and T.

3.2. Characterisation of the fish flesh at To and *in vivo* performances

At the beginning of the feeding trial, the sampled fish weighed 183.3 ± 29.6 g, were 24.50 ± 1.54 cm long and had CF of 0.97 ± 0.07 (mean ± SD). FY, HSI and VSI assumed the values of 63.13 ± 1.89, 0.73 ± 0.09 and 5.44 ± 0.59 % whole body weight, respectively. The values of pH, maximum shear force and percentage WHC of the fillets were 6.45 ± 0.09, 29.42 ± 2.00 N, and 92.96 ± 2.06%, respectively. The colour of the side of the skin showed the following values of the colour parameters: L*=54.27 ± 6.87, a*=-1.14 ± 0.70, b*=-0.13 ± 1.78, while the left fillets showed the following parameters: L*=52.46 ± 2.24, a*=0.40 ± 0.51, b*=1.98 ± 1.39. Water, ash and crude protein contents were 76.48 ± 1.08, 1.21 ± 0.33 and 19.08 ± 0.38 g/100 g sample, respectively. Cholesterol content was 52.74 mg/100 g of fillet. The FA profile of fillets at To is shown in Table S2.

During the whole trial, the rearing conditions remained in the optimal range for trout growth, as a matter of fact, the survival rate at the end of the experiment was very high (99.99%) and no relevant differences were noted between the three groups. The mean dissolved oxygen was 9.0 ± 0.49 mg/L and the water temperature fluctuated close to 12.1 ± 0.27°C (mean ± SD). The rearing density reached the maximum value of 11.5 kg/m^3 at the end of the trial. A difference in SGR, FCR and weight could be seen at the end of the trial (T2, Figure 1). A declining trend was noted in SGR and FCR going from C+ to T group, being C- intermediate and C+ showing a faster growth and a better FCR than T fish ($P<0.05$). The PER was not a discriminating factor between the groups.

3.3. Marketable traits and fillet physical characteristics at T1 and T2

After six weeks of feeding with the experimental diets (T1), fish belonging to the different dietary treatments did not show any difference as concerns marketable traits, pH, texture and WHC (data not shown). A longer time period of feeding though, i.e. after six weeks more of feeding (T2), T fish showed the highest and lowest values for FY and HSI, respectively, with a significant

difference against the C+ group ($P<0.05$), while C- group showed intermediate values for both these parameters (Table 2).

As concerns the colour of the skin and fillets of fish at the end of the 12-week feeding trial (Table 2), the redness index of the skin of T group was lower than that of C- group ($P<0.05$), and a pronounced yellow index was registered in C- and T fillets in comparison to C+ ($P<0.01$). The variation of colour (ΔE) between the fillets of the different dietary groups showed the following values: $\Delta E_{C+/C-}$ 1.59, $\Delta E_{C+/T}$ 1.94, and $\Delta E_{C-/T}$ 0.95.

3.4. Fillet chemical characteristics and estimation of indices of elongase and desaturase activity

Fillet proximate composition did not differ between the three dietary groups both at T1 (data not shown) and at T2 (Table 3); also, cholesterol content of fresh fillets at T2 was similar between the dietary treatments (Table 3).

Fillet fatty acid profile was profoundly affected by the experimental diets as early as after 6 weeks of administration. However, being the fatty acid profile found at T1 akin to the one found at T2, we depict the results obtained at T1 in Table S2, while those at T2 in Table 3. Mirroring the fatty acid profile of the diets, C- and T fillets resulted in a significantly higher PUFAn6 content compared to C+, while their PUFAn3 percentage significantly diminished. Consequently, PUFAn3/PUFAn6 ratio also decreased in C- and T groups. EPA and docosahexaenoic acid (DHA) also mirrored the composition of the diets. Interestingly, the content of C18:3n3 was comparable in C+ and T, while C- showed significantly higher values ($P<0.001$). By contrast to the dietary fatty acid profile, SFA content in T fillets was lower than both C+ and C- ($P<0.01$). Specifically, T fillets showed the lowest C14:0 ($P<0.001$, not shown) and C16:0 ($P<0.01$) contents in comparison to both C+ and C-; additionally, C18:0 content in C+ and T were analogous, while C- displayed significantly higher values ($P<0.05$) despite this FA was found higher in the T diet than in C- and C+ ones.

The results of the estimated indices of lipid metabolism are depicted in Table 4. Significant differences between the three dietary groups were revealed in all indices. Taking C- and T into consideration, the estimated activities of Δ9 desaturase (C18), Δ9 desaturase (C16+C18) and Δ5+Δ6 desaturase n3 were higher in T as compared to C- ($P<0.001$, $P<0.0001$, $P<0.0001$, respectively). On the other hand, the estimated activities of thioesterase, elongase, Δ9 desaturase (C16) and Δ5+Δ6 desaturase (n6) enzymes were similar between C- and T groups ($p>0.05$). Overall, T showed the highest Δ9 desaturase (C18), Δ9 desaturase (C16+C18) and Δ5+Δ6 desaturase n6 activities.

3.5. Oxidative status

As summarized in Table 5, parameters of the antioxidant capacity of fillets from fish fed for 12 weeks showed different trends. Specifically, ABTS and DPPH showed similar values between the groups differently fed, while FRAP value of the T group was significantly lower than that of C+ group ($P<0.01$), while C- group displayed intermediate value. TBARS assay indicated that the different diets influenced lipid peroxidation in fillets, showing that C- and T fillets were less prone to peroxidation in comparison to C+ ($P<0.05$).

4. Discussion

4.1 Fish performances, marketable and physical characterisation

The present study showed that the 5% dietary inclusion of a commercial blend of seaweeds tended to reduce rainbow trout growth performances. Differences were unveiled as early as after 6 weeks of feeding when it comes to colour of skin and flesh and of FA profile of fillets. Additional six weeks of feeding did not further change most of the parameters, although growth performances declined in T fish. In spite of the worsened growth performances, marketable traits could be considered acceptable for this species. For this reason, it seems reasonable to use macroalgae for a short period of time, for instance in finishing diets.

As a result of T diet administration, HSI decreased and FY increased ($P<0.05$) as compared to C+. It is commonly accepted that HSI provides an indication on the status of energy reserves and on the general metabolic activity. Researchers mainly noticed an increase in HSI following a decrease in feed availability, especially while studying seasonal variation of this index, indicating that energy usually allocated to tissue growth is destined to combat stressors (Craig, MacKenzie, Jones, & Gatlin III, 2000; Singh & Srivastava, 2015). Focusing on the impact of seaweed administration on fish HSI, Soler-Vila *et al*. (Soler-Vila et al., 2009) found that the HSI value decreased in rainbow trout fed for 12.5 weeks while increasing the substitution level of fishmeal and wheat starch. A decreasing trend was apparent and became significant at 10 or 15% dietary inclusion of the red alga *Porphyra dioica*. More recently, the incorporation of 5%, 15% or 25% of *Gracilaria cornea* or *Ulva rigida* meal in feed for sea bream (*Sparus aurata)* juveniles was tested after 70 days of administration (Vizcaíno et al., 2016). Results showed an inverse relationship between dietary inclusion of seaweeds and HSI value. Moreover, the HSI was significantly lower, compared to control, starting from the 5% inclusion level of both seaweeds. In the present study, the lowering of HSI, together with the reduced PER, could suggest the difficulty of fish to efficiently use feed components and metabolised energy. In this regards, a recent study carried out by Sotoudeh and Mardani (2018) on rainbow trout fry showed a diminished protease activity with increasing dietary *Gracilaria pygmaea* levels in feed. This outcome is in general agreement with Vizcaíno *et al.* (2016), who reported that the proteolytic activities in *S. aurata* fed *Ulva*- or *Gracilaria*-supplemented diets were lower than those of gilthead sea bream fed with the control diet.

Flesh pigmentation is one of the major quality attributes of salmonids (de Francesco et al., 2004) and it can be easily evaluated by instrumental analysis, which usually well reflects carotenoid deposition and concentration (Wathne, Bjerkeng, Storebakken, Vassvik, & Odland, 1998). Carotenoids, such as β-carotene, astaxanthin and yellow xanthophylls, especially lutein and zeaxanthin, are mainly responsible for flesh colouration. These molecules are widespread both in terrestrial vegetables and seaweeds (Wells et al., 2017). For this reason, in the present study, the variation of colour in fish tissues can be attributed to the different proportions of the vegetable ingredients in the experimental diets as well as to the seaweed inclusion. In this regard, the colour values that were similar between C- and T diets, which both resulted in higher a* and b* values than those of C+ diet, supported the idea that the vegetal ingredients were the main source of pigments. These differences also corroborated results obtained for the skin and the muscle of fish, where only a slight difference in the a* value of the skin emerged between C- and T. Overall, a* and b* indices of the skin and of the muscle of trout were significantly affected by the dietary treatments, thus confirming the findings of Araújo *et al.* (Araújo et al., 2016), who showed that different pigments preferentially deposit in skin or muscle. Indeed, rainbow trout skin was found to be the principal accumulation site for β-carotene, positively correlated with a* index, while muscle mainly stores lutein and zeaxanthin, whose concentrations are positively correlated with b* value (Araújo et al., 2016).

Colour differences (ΔE) underlined that no difference in colour could be perceived between C- and T ($\Delta E < 1$), while C+ differed from both C- and T ($1<\Delta E<3.5$) (Mokrzycki & Tatol, 2011). In summary, the present results revealed that 5% soybean substitution with a commercial blend of seaweeds, in a vegetable rich diet, is not enough to deeply modify skin and muscle colourations of trout, nor to convey a redder flesh pigmentation, as claimed by Araújo *et al*. (2016) and Soler-Vila *et al*. (2009).

Recently, authors have found that changes in the dietary protein sources, such as type and quantity, did not affect textural attribute of rainbow trout fillets. Indeed, Borgogno *et al*. (2017) verified that the Warner-Bratzler shear force registered for rainbow trout muscle was unaffected with increasing substitution levels of fishmeal with insect meal. However, the performed sensory analysis revealed that differences in feed composition were perceived by trained panellist in terms of fillet textural attributes (tenderness, juiciness, fibrousness). Since no effect of the experimental diet on shear force emerged in the present trial, further studies would be useful to understand if texture might be perceived as different by a trained panel or if liking judgements of fillets by consumers might be altered by the composition of the feed utilised for feeding the fish.

Another possible explanation of the decreased HSI might be a reduced deposition of lipid in the liver, as previously found in *O. mykiss* fed on a diet containing 10% *Ulva* meal (Güroy et al., 2013). Supporting this assertion, Dantagnan *et al*. (2009) highlighted that, in *O. mykiss* juveniles, the lipid utilization can be enhanced by macroalgae meal in diets*,* thus resulting in a lower

amount of lipid deposition in tissues than in the no-added diet. In agreement with Dantagnan *et al*. (2009), a marginal effect (P=0.053) on fillet total lipid was also found in the present study.

4.2 Fillet chemical composition and estimated lipid metabolism

Concerning the overall proximate composition of the fillets, the absence of a significant effect of the diet aligns with previous studies that considered the 5% inclusion level (dry matter) of *Gracilaria vermiculophylla* and *Porphyra dioica* in feed for rainbow trout (Araújo et al., 2016; Soler-Vila et al., 2009), *Gracilaria cornea* and *Ulva rigida* in feed for gilthead sea bream juveniles (*Sparus aurata*) (Vizcaíno et al., 2016), and *Sargassum horneri* in feed for juvenile turbot (*Scophthalmus maximus*) (Wang et al., 2018). Nevertheless, the lower lipid content in T samples was of notice, since a numerical difference near to the significant threshold was registered (the calculated p-value was 0.054). In literature, the lipid source and its level of inclusion in aquafeeds are the most reported modulators of lipase activity (Morais et al., 2004), but the addition of different seaweed species at various concentrations also seemed to modulate the activity of this enzyme (Peixoto et al., 2016). Indeed, intestinal lipase activity of European sea bass (*Dicentrarchus labrax*) fed with diets supplemented with no seaweed or with *Gracilaria* spp., *Ulva* spp., and *Fucus* spp. at 2.5 or 7.5% and a mix of the three (each at 2.5% inclusion percentage) for 84 days was not significantly different between the different dietary treatments groups except for fish fed *Ulva* spp., which had the lowest lipase activity between all the treatments, with a significant difference compared to fish fed the mix of the three seaweeds. These results encourage researchers to enrich the literature with additional results from trial focusing on the effects of macroalgae on rainbow trout lipid deposition.

It is a matter of fact that the level of PUFAn3 in the fillets of farmed fish has dramatically declined in the recent years due to the replacement of PUFAn3-rich fish oil and fish meal with vegetable sources rich in MUFA and PUFAn6 (Storebakken, Refstie, & Ruyter, 2000). In this regard, the use of seaweeds, another marine source rich in PUFAn3 (Wells et al., 2017), could counteract the high MUFA and PUFAn6 content of vegetable sources contained in feed ingredients. As exposed in Table 1, PUFAn6 and SFA mainly represented the fatty acid composition of C- diet, hence in line with the trend registered during the last decade. Moreover, the analogy between the fatty acid profiles of C- and T diets showed that the 5% inclusion of the commercial blend of seaweeds was not sufficient to induce a substantial modification of the fatty acid composition of the diet, especially concerning PUFAn3 content.

Nevertheless, recent studies have enquired the possibility that dietary seaweeds enhance PUFAn3 in fish muscle (Dantagnan et al., 2009; Güroy et al., 2013; Wilke et al., 2015). Although the fillet FA profile results did not manifestly back up this hypothesis, the estimated indices of lipid metabolism suggested that

the commercial seaweed blend modulated the activity of certain enzymes. The Δ9 desaturase activity on stearic acid was significantly higher in T fish in comparison to C- fish ($P<0.001$). It seems also noteworthy to comment on the Δ5-Δ6 desaturase activity carried out on the n3 series, namely, the index increased in the T group in comparison to the C- group ($P<0.0001$). The differences between C- and T groups suggest that the enzymes involved in the desaturation of long chain and very long chain FAs were modulated by the dietary inclusion of seaweeds. As expected, the estimated metabolic indices were significantly different between C+ and C- and this is easily explained by revising dietary FA composition. In fact, the considerable difference in FA profile between of C- and C+ diets likely induced a regulation of fish elongase and desaturase enzymatic activities, as well documented in other studies on Atlantic salmon where diets with different amounts of PUFAn3 were compared (Stubhaug, Tocher, Bell, Dick, & Torstensen, 2005; Turchini & Francis, 2009).

Noticeably, some slight divergence between feed and fillet FA profiles was found regarding SFA. While the T diet contained the same amount of SFA as the C- diet, this was not the case for the fillets from fish fed with these diets. Despite the paucity of research highlighting a decrease of SFA content n fish fed with algae, experiments on rats fed with ethanolic extracts of *Ecklonia stolonifera* (Yoon, Kim, Chung, & Choi, 2008) or with fucoidan polysaccharide sulfuric acid ester from *Laminaria japonica* Aresch (Huang, Wen, Gao, & Liu, 2010) registered a significant reduction in triglycerides (TGs) in hyperlipidemic rat plasma. In addition, an *in vitro* study supported this thread, observing that polysaccharides extracted from different algae induced hypolipidemic activity on human cell lines insignificantly different as compared to the reference drug Fluvastatin (Matloub et al., 2015). Reminding that it was found that *Ulva* spp. lowered the activity of lipase (Peixoto et al., 2016), responsible of TG and not phospholipid hydrolysis, and since TGs, in comparison to phospholipids, generally contain a lower proportion of PUFA and a higher proportion of SFA, the reduction of SFA in the total lipid extract of fillets could be explained by a possible reduction in lipase activity, induced by the dietary seaweed blend. However, more studies about the lipid metabolism of fish fed seaweeds are strongly suggested.

4.3 Fillet oxidative status

Finally, there is a very broad literature on marine algae as sources of antioxidant compounds (Wells et al., 2017), which play an important role in preventing lipid oxidation by acting in several ways, such as radical scavengers, chelating agent, etc. In the present study, the similarities of the results on the total antioxidant capacity (monitored by ABTS, DPPH, and FRAP assays) and lipid peroxidation products (TBARS) between fillets belonging to the T and C- groups suggested that T diet did not carry a specific antioxidant ability in comparison to the C- diet. This result goes in the same direction as previous studies which did not find differences in hepatic TBARS levels of sea bass juveniles fed 6 practical diets supplemented either with or without *Gracilaria* spp., *Ulva* spp. or *Fucus* spp.

at 2.5 or 7.5% levels, or a mix of the three seaweeds (each supplemented at 2.5%) (Peixoto et al., 2016). Moreover, Sotoudeh and Mardani (2018) have recently quantified TBARS content in the liver of rainbow trout fry fed diets supplemented with 0, 30 and 60 g/kg of *Gracilaria pygmaea*, finding that it was not significantly affected by the different diets.

5. Conclusions

Seaweeds are a promising source of not only nutritive but also nutraceutical compounds and research in this field should be warmly encouraged, as to delve into the effects of different algal species on fish metabolism. The present study showed that most marketable and physical parameters of rainbow trout fed with a low dietary inclusion level of a commercial blend of seaweeds were not negatively affected, while fatty acid profile is more of a concern. On the other hand, the study carried out highlighted an interesting lowering effect on SFA content and an increase in the estimated desaturase activity. Studies in this specific field are also highly encouraged.

Declaration of interest

All authors declare no conflict of interest.

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Table 1. Formulation, proximate composition and FA groups content of the extruded experimental diets.

	C+	C-	T
Ingredients (% as fed)			
Fishmeal 999	53.2	6.2	6.2
CPSP 90 IDR	6.0	6.2	6.2
Wheat gluten	8.5	10.3	10.3
Corn gluten	0.0	12.4	12.4
Soy protein concentrate	8.5	10.3	10.3
Commercial blend of seaweeds	0.0	0.0	5.2
Soy meal extract (48%)	0.0	25.8	20.6
Fish oil	15.1	8.2	8.2
Rapeseed oil	6.0	11.9	11.9
Idropalm	2.4	3.6	3.6
Vitamin premix	0.1	0.1	0.1
Oligomineral premix	0.1	0.1	0.1
Dicalcium phosphate	0.0	2.6	2.6
DL-Methionine	0.0	0.5	0.5
Lysine-HCL	0.0	1.5	1.5
Betaine	0.0	0.3	0.3
Total	100.0	100.0	100.0
Fish protein (% of crude protein)	70	10	10
Fat coming from fish (% of crude fat)	70	35	35
Chemical composition			
Crude protein (% on a.i. basis)	41.2	41.1	41.0
Crude fat (% on a.i. basis)	24.4	24.2	24.3
Fatty acid groups (g/100 g total FAME)			
ΣSFA	22.856	27.433	29.945
ΣMUFA	42.892	40.190	40.666
ΣPUFAn6	14.959	19.920	18.119
ΣPUFAn3	18.472	11.936	10.774
PUFAn3/PUFAn6	1.24	0.60	0.60

Table 2. Growth performances of fish and marketable and physical characteristics of fresh fish and fillets at T2 (12-week long feeding trial).

		C+	C-	T	*P-value*	*RMSE*
BW at T2, g		474.97[a]	442.27[b]	416.00[c]	*<0.01*	*58.62*
Standard length, cm		30.33	29.05	29.68	*ns*	*1.05*
CF		1.25	1.27	1.18	*ns*	*0.09*
FY, %		62.75[b]	64.05[ab]	64.51[a]	*<0.05*	*1.13*
HSI, %		1.27[a]	1.15[ab]	0.99[b]	*<0.05*	*0.17*
VSI, %		8.85	8.01	7.52	*ns*	*1.03*
pH		6.42	6.46	6.48	*ns*	*0.05*
Maximum Shear Force, N		28.72	28.46	30.13	*ns*	*3.80*
WHC, %		92.75	92.56	93.39	*ns*	*2.53*
Skin colour	**L***	59.95	59.53	61.74	*ns*	*4.01*
	a*	-1.57[ab]	-1.22[a]	-1.84[b]	*<0.05*	*0.39*
	b*	-0.64	-1.58	-1.28	*ns*	*1.23*
Fillet colour	**L***	49.32	48.63	49.42	*ns*	*1.30*
	a*	-0.35	-0.36	-0.51	*ns*	*0.62*
	b*	2.92[b]	4.60[a]	5.20[a]	*<0.01*	*1.02*

a, b as superscript letters indicate significant difference among groups; *ns*: not significant, P>0.05.

RMSE: root mean square error; BW: whole body weight; CF: condition factor; FY: fillet yield; HSI: hepatosomatic index; VSI: viscerosomatic index; WHC: water holding capacity.

Table 3. Chemical composition (g/100g fresh tissue), cholesterol content (mg/100g fresh tissue) and fatty acid profile (g of FAME/100 g total FAME) of fillets at T2 (12-week long feeding trial).

	C+	C-	T	*P-value*	*RMSE*
Moisture	73.59	73.76	74.35	*ns*	*0.60*
Ash	1.28	1.28	1.26	*ns*	*0.03*
Crude protein	19.88	20.08	20.18	*ns*	*0.29*
Cholesterol	49.663	48.265	48.670	*ns*	*2.835*
Total lipids	5.60	4.94	4.49	*ns* (0.0536)	*0.72*
Fatty acids					
C16:0	13.369^{a}	13.640^{a}	12.900^{b}	*<0.01*	*0.334*
C18:0	4.605^{b}	5.138^{a}	4.720^{b}	*<0.05*	*0.274*
C18:1n9	32.441^{b}	32.853^{b}	34.146^{a}	*<0.05*	*0.996*
C18:2n6	14.493^{b}	17.843^{a}	17.118^{a}	*<0.001*	*0.698*
C18:3n3	3.388^{b}	3.743^{a}	3.418^{b}	*<0.001*	*0.132*
C20:5n3	3.335^{a}	2.020^{b}	2.214^{b}	*<0.001*	*0.214*
C22:6n3	11.796^{a}	9.183^{b}	10.008^{b}	*<0.01*	*1.006*
sum EPA+DHA	15.131^{a}	11.203^{b}	12.222^{b}	*<0.01*	*1.586*
SFA	20.59^{a}	20.80^{a}	19.56^{b}	*<0.01*	*0.52*
MUFA	40.65	39.81	40.95	*ns*	*1.13*
PUFAn6	17.48^{b}	22.19^{a}	21.57^{a}	*<0.001*	*0.76*
PUFAn3	20.67^{a}	16.73^{b}	17.46^{b}	*<0.001*	*1.17*
PUFAn3/PUFAn6	1.18^{a}	0.75^{b}	0.81^{b}	*<0.001*	*0.07*

The following FAs were used for calculating the classes of FAs but they are not listed because below 3% of total FAME: C12, C13, C14, C14:1n5, C15, C15iso, C15anteiso, C16iso, C16:1n7, C16:1n9, C16:2n4, C16:3n4, C16:4n1, C17, C17:1, C18:1n7, C18:2n4, C18:3n6, C18:3n4, C18:4n1, C20, C20:1n11, C20:1n9, C20:1n7, C20:2n6, C20:4n6, C20:3n3, C20:4n3, C22, C22:1n7, C22:1n11, C22:2n6, C21:5n3, C22:4n6, C22:5n6, C22:5n3, C24.

a, b as superscript letters indicate significant difference among groups; *ns*: not significant, P>0.05.

RMSE: root mean square error.

Table 4. Estimated indices of lipid metabolism in fresh fillet at T2 (12-week long feeding trial).

	C+	C-	T	*P-value*	*RMSE*
Thioesterase	7.945^{b}	10.810^{a}	10.782^{a}	*<0.0001*	*0.419*
Elongase	0.344^{b}	0.376^{a}	0.366^{ab}	*<0.05*	*0.019*
Δ9 desaturase (C16)	17.460^{a}	14.704^{b}	14.557^{b}	*<0.0001*	*0.903*
Δ9 desaturase (C18)	88.328^{a}	87.268^{b}	88.535^{a}	*<0.001*	*0.476*
Δ9 desaturase (C16+C18)	67.678^{b}	66.639^{c}	68.670^{a}	*<0.0001*	*0.573*
Δ5+Δ6 desaturase n6	9.860^{b}	10.881^{a}	11.445^{a}	*<0.01*	*0.721*
Δ5+Δ6 desaturase n3	82.247^{a}	75.521^{c}	78.735^{b}	*<0.0001*	*1.713*

a, b, c as superscript letters indicate significant difference among groups; *ns*: not significant, *P*>0.05.

RMSE: root mean square error.

Table 5. Oxidative status of fresh fillets at T2 (12-week long feeding trial). ABTS, DPPH, and FRAP values are expressed as mmol Trolox-eq/kg fillet; TBARS values are expressed as mg MDA-eq/kg fillet.

	C+	C-	T	*p-value*	*RMSE*
ABTS	0.417	0.435	0.404	*ns*	*0.099*
DPPH	0.106	0.130	0.122	*ns*	*0.017*
FRAP	0.105^{a}	0.098^{ab}	0.091^{b}	*<0.01*	*0.006*
TBARS	0.505^{a}	0.301^{b}	0.299^{b}	*<0.05*	*0.109*

a, b as superscript letters indicate significant difference among groups; *ns*: not significant, P>0.05.

RMSE: root mean square error.

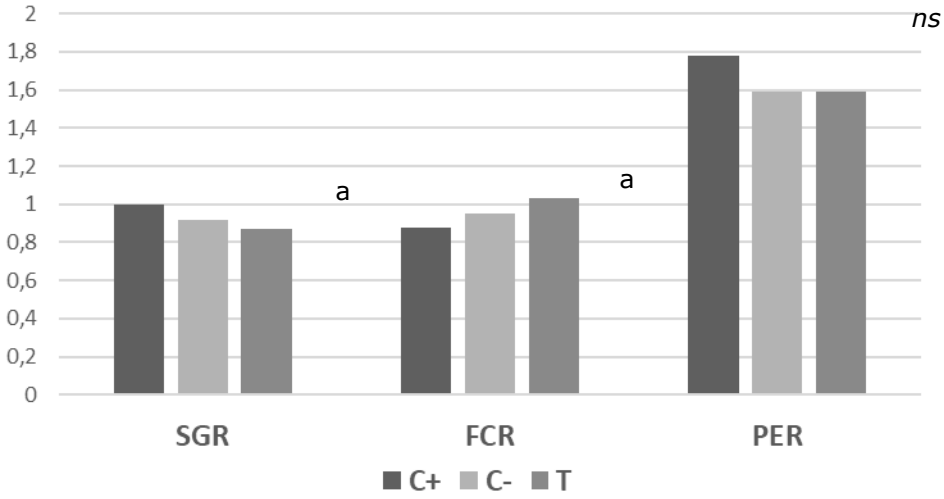

Figure 1. Growth performances of fish during the feeding trial.

a, b as superscript letters indicate significant difference among groups, P<0.05; *ns*: not significant, P>0.05.

Paper 6

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Partial Dietary Inclusion of *Hermetia illucens* (Black Soldier Fly) Full-Fat Prepupae in Zebrafish Feed: Biometric, Histological, Biochemical, and Molecular Implications

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Abstract

Due to minimal environmental impact, compared to most conventional feed commodities, insects deserve a growing attention as candidate ingredients for aquafeeds. This study tested, for the first time during zebrafish larval rearing, the effects of an increasing replacement (0%–25%–50%) of fish meal by black soldier fly (BSF) full-fat prepupae meal. All diets were formulated to be isonitrogenous and isolipidic. A multidisciplinary approach, including biometrics, histology, gas chromatography–mass spectrometry, and molecular analyses, was applied to better understand the biological responses of larval zebrafish to the different partial inclusions of BSF in the feed. Generally, results are promising, but a 50% of BSF meal inclusion in the diet affected both lipid composition and accumulation in the larvae.

Keywords: black soldier fly, gene expression, fatty acid profile, insect diet, aquaculture

Introduction

FISH MEAL AND FISH OIL represent the ideal ingredients for carnivorous fish diets due to their excellent nutritive properties, including high-protein content and adequate amino acid and fatty acid (FA) profile. Usually, high digestibility and palatability and the absence of antinutritional factors characterize this ingredient, ensuring optimal growth and health of cultured fish.[1]

Since fish meal and fish oil represent a crucial part of the current industrially produced diets,[2,3] the use of wild fish to feed farmed fish places direct pressures on fishery resources. In addition, fish meal and fish oil prices have dramatically risen in the past years,[3] undermining the profitability of many aquaculture enterprises. Consequently, searching for alternatives to fish meal and oil represents a primary issue to be addressed to have a more sustainable aquaculture production.[4]

Over the last decades, research has focused on testing different alternative ingredients to be used in aquafeed, including oilseeds, animal by-products, and microalgae. Most of the studies have focused on a possible application of vegetal proteins in fish feeds. Unfortunately, only a partial substitution is feasible because vegetal proteins may have unbalanced amino acid profile, poor protein digestibility, and antinutritional substances content.[5] Furthermore, oilseed cultivation is often based on deforestation and high water consumption, which led to additional environmental issues.[6]

Because of their adequate nutritional profile, their high content in pigments, antioxidants, and bioactive compounds, microalgae have been investigated as an alternative feed ingredient.[7] However, despite their advantages, the use of microalgae faces severe barriers due to their current high costs and the difficulty in producing, concentrating, and storing them.[7]

Scientists are now focusing on insects as a new protein source for animal feed.[8] Insects are an excellent source of nutrients,[9,10] they are highly productive, and are able to transform low-quality organic waste and manure into highly nutritious substances, playing a central role in the development of a circular bioeconomy.[10,11] Furthermore, the European Commission recently gave a boost to insect production, allowing the use of processed insects in feed for aquaculture.[12] It is clear that partially substituting fish meal with insect meal will become a reality in animal farming in the EU.

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Partial dietary inclusion of *Hermetia illucens* (black soldier fly) full-fat prepupae in zebrafish feed: biometric, histological, biochemical and molecular implications.

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Abstract

Due to minimal environmental impact, compared to most conventional feed commodities, insects deserve a growing attention as candidate ingredients for aquafeeds. The present study tested, for the first time during zebrafish larval rearing, the effects of an increasing replacement (0-25-50%) of fish meal by Black Soldier Fly (BSF) full-fat prepupae meal. All diets were formulated to be isonitrogenous and isolipidic. A multidisciplinary approach, including biometrics, histology, gas chromatography mass spectrometry and molecular analyses was applied in order to better understand the biological responses of larval zebrafish to the different partial inclusions of BSF in the feed. Generally, results are promising, but a 50% of BSF meal inclusion in the diet affected both lipid composition and accumulation in the larvae.

Key words

Black soldier fly, gene expression, fatty acid profile, insect diet, aquaculture

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INTRODUCTION

Fish meal and fish oil represent the ideal ingredients for carnivorous fish diets due to their excellent nutritive properties, including high-protein content and adequate amino acid and fatty acid profile. Usually, high digestibility and palatability and the absence of anti-nutritional factors characterize this ingredient, ensuring optimal growth and health of cultured fish.[1]

Since fish meal and fish oil represent a crucial part of the current industrially produced diets,[2,3] the use of wild fish to feed farmed fish places direct pressures on fishery resources. In addition, fish meal and fish oil prices have dramatically risen in the past years[3] undermining the profitability of many aquaculture enterprises. Consequently, searching for alternatives to fish meal and oil represents a primary issue to be addressed in order to have a more sustainable aquaculture production.[4]

Over the last decades research has focused on testing different alternative ingredients to be used in aquafeed, including oilseeds, meat by-products, and microalgae. Most of the studies have focused on a possible application of vegetal proteins in fish feeds. Unfortunately, only a partial substitution is feasible because vegetal proteins may have unbalanced amino acid profile, poor protein digestibility and anti-nutritional substances content.[5] Furthermore, oilseed cultivation is often based on deforestation and high water consumption which led to additional environmental issues.[6]

Because of their adequate nutritional profile, their high content in pigments, antioxidants and bioactive compounds, microalgae have been investigated as an alternative feed ingredient[7]. However, despite their advantages, the use of microalgae faces severe barriers due to their current high costs and the difficulty in producing, concentrating and storing them.[7]

Scientists are now focusing on insects as a new protein source for animal feed.[8] Insects are an excellent source of nutrients,[9,10] they are highly productive and are able to transform low-quality organic waste and manure into highly nutritious substances, playing a central role in the development of a circular bioeconomy.[10,11] Furthermore, the European Commission recently gave a boost to insect production allowing the use of processed insects in feed for aquaculture.[12] It is clear that partially substituting fish meal with insect meal will become a reality in animal farming in the EU.

Hermetia illucens (L.) (Diptera, Stratiomydae) (black soldier fly, BSF) is one of the most promising insect species because it shows an essential amino acid pattern similar to that of fish meal.[9] However, the fatty acid profile of insects does not always meet the nutritional requirements of fish since insects are normally rich in saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), rather than polyunsaturated ones. Another important consideration about the use of insects in aquafeed is the presence of chitin into their exoskeleton. Some fish species are capable to break down chitin glycosidic bond

using endogenous enzymes (chitinases) or by the activity of chitinolytic bacteria.[13,14] However, the presence of chitin in the diet can reduce food intake, digestibility and nutrient absorption in cultured fish because of their limited digestive capacity for this polysaccharide.[15]

Studies have shown that fish feeds can include up to an intermediate amount of *H. illucens* without impairing growth performance nor feed digestibility.[16–19]

Zebrafish (*Danio rerio*) is one of the most studied experimental models in biomedical sciences, developmental biology, genetics, toxicology and aquaculture [20,21] due to its high reproductive rate and to the abundant information that has recently become available from genomic sequencing. To the best of our knowledge, there is no study performed on the larval development of zebrafish using partially substituited insect based diets. Improvements in larval diet efficiency and formulation is now a priority and the desirable goals are a reduction or even a complete substitution of unsustainable marine protein and lipid feedstuff with more sustainable protein and lipid sources like those produced through insect rearing. A previous study by Vargas and collaborators (2018)[22] evidenced that a 100% BSF diet did not impair zebrafish larval development over a 21 days experiment. However, it is well established that over longer periods of time, insect meal could reduce fish growth and welfare because of a substantial high content in chitin[23] in addition to the deficiency in some essential amino acid.[22] For these reasons there is a strong need to test well balanced formulated diets with lower insect meal inclusions.

The present study investigates for the first time and through a multidisciplinary approach, including biometrics, histology, gas chromatography mass spectrometry and molecular analyses, the biological effects on zebrafish larval development of different partial inclusions of BSF meal in formulated isonitrogenous and isolipidic diets.

MATERIALS AND METHODS

Ethics

All procedures involving animals were conducted in line with Italian legislation on experimental animals and were approved by the ethics committee of the Università Politecnica delle Marche. Optimal rearing conditions (see further section for details) were applied throughout the study, and all efforts were made to minimize animal suffering by using an anesthetic (MS222; Sigma Aldrich, Milano, Italy).

Diets

Three experimental diets including increasing levels of BSF were formulated and produced at the facilities of the University of Udine. BSF prepupae were purchased from a commercial company (Smart Bugs s.s. company, Ponzano Veneto, TV, Italy) where the insects were reared on a substrate composed by

corn meal and fruit and vegetable mixture (50:50). Prepupae, once collected, were frozen (-80°C), freeze-dried and minced and through the use of liquid nitrogen.

The diets were formulated to be isonitrogenous (CP 40%) and isolipidic (EE 18%) with BSF full-fat prepupae replacing 25% (Group A) and 50% (Group B) of the fish meal of the basal diet (Control), respectively. Diets were analysed for proximate composition (AOAC, 1998) and gross energy content measured by an adiabatic bomb calorimeter (IKA C7000, Werke GmbH and Co., Staufen, Germany). The ingredient composition, proximate analysis and energy content of the experimental diets is shown in Table 1.

Fish

Zebrafish AB embryos were spawned and maintained 48h in a Tecniplast system (Varese, Italy), subjected to the following conditions: pH 7.0, NO_2 and NH_3 concentrations < 0.01 mg/L, NO_3 concentration < 10 mg/L, photoperiod 12L/12D. After this first period, embryos were gently collected, counted under a stereomicroscope (Leica Wild M3B, Leica Microsystems, Nussloch, Germany) and randomly divided in three experimental groups according to the three test diets.

Experimental design

Zebrafish larvae were kept in nine 20 L tanks to set up the three experimental dietary treatments, each of 1500 fish (500 fish per tank). Fish were fed as follows: Control group, larvae fed the fish meal/oil based diet; Group A, larvae fed the diet including 25% of BSF full-fat prepupae meal; Group B, larvae fed the diet including 50% of BSF full-fat prepupae meal.

Zebrafish larvae were maintained in 20 L tanks fed the experimental diets (2% body weight, BW) twice a day.[24] The water in larval tanks (same chemical-physical characteristics of the parent's tank) was gently replaced ten times a day by a dripping system. The sides of the tank were covered with black panels to reduce light reflection).[25,26] Larvae were sampled at 7, 14 and 21 dps, euthanized with an excess of anesthetic (MS222 1g/L, Sigma Aldrich, Milano, Italy) and properly stored for further analyses. All groups were fed (one feeding in the morning) on the rotifer *Brachionus plicatilis* (5 ind/mL) from 5 to 10 days post-spawning (dps) according to Lawrence *et al*. (2012).[27]

Biometry and survival

For biometric measurements, 10 larvae per tank (30 per group) were randomly collected from the different tanks, individually measured and bulk-weighed at each sampling time. The standard length was determined through the use of a sliding calliper (Measy 2000 Typ 5921, Swiss; precision: 0.1 mm) and the dry weight (calculated in pools of ten larvae) with an OHAUS Explorer (Greifensee, Switzerland) analytical balance (precision: 0.1 mg). Survival was determined by counting the remaining larvae in each tank at the end of the experiment (21 dps).

Lipid content and Fatty acid composition

The experimental diets and the larval fish samples collected at 21 dps (Control, Group A and Group B) were analyzed, in duplicate, for lipid content and fatty acid composition. Diets and fish larvae were minced, homogenized (homogenizer MZ 4110, DCG Eltronic, Monza, Italy) and freeze-dried (Edwards EF4, Crawley, Sussex, England) and lipid extraction was carried out on lyophilized powders following a microwave-assisted extraction (MAE).[28,29] Fatty acid methyl esters (FAMEs) were prepared according to Truzzi *et al.* (2017),[29] using the methyl ester of nonadecanoic acid (19:0; Dr. Ehrenstorfer GmbH, Augsburg, Germany) as internal standard. FAMEs were determined by an Agilent-6890 GC System (Milano, Italy) coupled to an Agilent-5973N quadrupole Mass Selective Detector (MSD) (Milano, Italy). A CPS ANALITICA CC-wax-MS (30 m × 0.25 mm ID, 0.25 µm film thickness) capillary column was used to separate FAMEs. Instrumental conditions for the study matrices were set up, according to Truzzi *et al*. (2017).[29] The mass fraction of fatty acids, expressed as mg/g tissue dry weight, was measured against the internal standard. For each sample, at least three runs were performed on the GC-MS. The precision of the proposed method was evaluated for the studied matrices as in Truzzi *et al*. (2014);[30] the intra-day and inter-day precision were, for major FAs, < 4% and < 7%, respectively, indicating a good repeatability of the analyses (data not shown). For FAs with a percentage <1% *vs* total FAs, intra-day and inter-day precision ranged from 5% to 20%, and from 8 to 25%, respectively. The estimated limits of detection (LOD) and limits of quantification (LOQ), calculated as in Truzzi *et al*. (2014),[31] ranged for each FAME from ~4 µg mL^{-1} to ~22 µg mL^{-1}, and from ~13 µg mL^{-1} to ~66 µg mL^{-1}, respectively.[29]

Histology

Five zebrafish larvae, in triplicate, randomly collected at each sampling time from the tanks of the three dietary treatments were fixed by immersion in Bouin's solution (Sigma-Aldrich, Milano, Italy) and stored at 4°C for 24h. Larvae were washed three times with ethanol (70%) for ten minutes and preserved in the same ethanol solution. Larvae were then dehydrated in crescent ethanol solutions (80, 95 and 100%), washed with the clearing agent "Histo-Clear" (Bio-Clear, Bio Optica, Milano, Italy) and embedded in paraffin (Bio-Optica). Solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS, Nussloch, Germany) and 5 µm sections were stained with Mayer hematoxylin and eosin Y (Sigma-Aldrich, Milano, Italy). Sections were observed using a Leica MD750 (Nussloch, Germany) optical microscope connected with a Leica ICC50 HD (Nussloch, Germany) camera.

RNA extraction and cDNA synthesis

Total RNA extraction from 20 zebrafish larvae (in triplicate), randomly collected from the different tanks at each sampling time, was optimized using RNAzol® RT reagent (SIGMA-ALDRICH®, R4533) following the manufacturer's instructions. Total RNA extracted was eluted in 20 µl of RNase-free water (Qiagen). Final RNA concentration was determined by the NanoPhotometer® P-Class (Implen, München, Germany). RNA integrity was verified by GelRed™ staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stored at -80°C

until use. Finally, 3 µg of RNA were used for cDNA synthesis, employing the High Capacity cDNA Reverse Transcription Kit (Bio-Rad) following the manufacturer's instructions.

Real-Time PCR

PCRs were performed with SYBER Green in an iQ5 iCycler thermal cycler (both from Bio-Rad) in triplicate. Reactions (10 µL) were set on a 96-well plate by mixing for each sample 1 µL cDNA diluted 1:20, 5 µL of 2x concentrated iQ™ Syber Green as the fluorescent intercalating agent, 0.3 µM forward primer, and 0.3 µM reverse primer. The thermal profile for all reactions was: 3 min at 95°C, followed by 45 cycles of 20 s at 95°C, 20 s at 60°C and 20 s at 72°C. Fluorescence was monitored at the end of each cycle. Dissociation curve analysis showed a single pick in all cases.

Relative quantification of the expression of genes involved in fish growth (*igf1*, *igf2a* and *mstnb*), stress response (*hsp70.1* and *nr3c1*), enzymatic hydrolysis of chitin (*chia.1*, *chia.2*, *chia.3*, *chia.4*, *chia.5* and *chia.6*), long-chain polyunsaturated fatty acid biosynthesis (*elovl2*, *elovl5* and *fads2*) and immune response (*tnfa* and *il6*) was performed using *arp* and *rpl13* as housekeeping genes to standardize the results (Table 2). Data were analyzed using the iQ5 optical system software version 2.0, including Genex Macro iQ5 Conversion and Genex Macro iQ5 files.

Amplification products were sequenced and homology was verified. No amplification product was detected in negative controls and no primer-dimer formation was found in control templates. Data were analyzed using the iQ5 optical system software version 2.0, including Genex Macro iQ5 Conversion and Genex Macro iQ5 files (all from Bio-Rad). Modification of gene expression was reported with respect to controls. Primer sequences were designed using Primer3 (210 v. 0.4.0) starting from zebrafish sequences available in ZFIN.

Statistical analysis

Survival data were analyzed by one-way ANOVA, with diet as the explanatory variable. The content and the fatty acid composition of diets and zebrafish larvae were analyzed by both t-test and one-way ANOVA. Standard length, dry weight and real-time PCR data were analyzed by two-way ANOVA, with both diet and dps as the explanatory variables. All ANOVA tests were followed by Tukey's post-hoc test. The statistical software package Prism5 (GraphPad Software) was used. Significance was set at $p < 0.05$ and all results are presented as mean ± SD.

RESULTS

Biometry and survival

Considering survival, no significant differences ($p>0.05$) were observed among the experimental groups. Group A reached the highest survival value (74±13%), while Control and Group B showed a 62±15% and 60±5% survival, respectively (Fig. 1).

Considering the standard length (Fig. 2a), no significant differences ($p>0.05$) were detected among the experimental groups at 7 and 14 dps. At the end of the experiment (21 dps), Control showed a significant lower value ($p<0.05$) in standard length respect to Group B (3.8±0.2 and 5.4±0.4 mm, respectively), while no significant differences were observed among Group A (4.9±0.3 mm) and the other groups. Finally, considering dry weight (Fig. 2b), no significant differences ($p>0.05$) were evident among the experimental groups at each sampling time, except for Group B that, at 21 dps, presented the highest value (3.8±1.0 mg).

Fatty acid content and composition

Diets. Table 3 reports the FAs composition (% FAMEs) of the experimental diets. The most relevant SFA was the palmitic acid (16:0), while lauric acid (12:0) increased significantly according to the dietary BSF meal inclusion. Oleic acid (18:1n9) was the most abundant MUFA in all dietary treatments. Control diet showed the highest amount of DHA (22:6n3; 134.2 mg/g) and EPA (20:5n3; 68.5 mg/g) which significantly decreased in diet A (42.4 and 82.4 mg/g, respectively), and B (31.2 and 59.7 mg/g, respectively).

The effect of the dietary inclusion of the BSF meal on selected groups of fatty acids of the experimental diets is presented in Figure 3. Considering MUFA content, no significant differences ($p>0.05$) were detected among the tested diets. Differently, the increasing inclusion levels of BSF full-fat prepupae meal resulted in a significant increase ($p<0.05$) of dietary SFA content (337.6, 421.3 and 482.4 mg/kg for diet Control, A and B, respectively) and a parallel decrease in PUFA (polyunsaturated fatty acids) content (330.0, 230.0 and 185.0 mg/kg for diet Control, A and B, respectively; $p<0.05$). Regarding PUFA composition, a significant reduction ($p<0.05$) in n3 PUFA content was observed with the increasing dietary inclusion of BSF meal, while no significant differences ($p>0.05$) were detected among the tested diets regarding both n6 and n9 fatty acids. Finally, as concerns n6/n3 ratio, no significant differences ($p>0.05$) were observed between Control (0.50±0.11) and Group A (0.71±0.12) diets, while in Group B (0.85±0.12) diet the ratio was significantly ($p<0.05$) higher respect to Control.

Zebrafish larvae. Table 4 reported the fatty acid composition of zebrafish larvae. Considering SFA composition in the three experimental groups, the predominant fatty acid was the palmitic acid (16:0), followed by stearic acid (18:0). Both these FAs did not present significant differences ($p>0.05$) among the larvae. Group B fish showed a significant ($p<0.05$) higher content of lauric (12:0) and myristic (14:0) acids with respect to Group A and Control fish. The most represented MUFA was oleic acid (18:1n9) and its content did not significantly ($p>0.05$) vary among the experimental zebrafish larvae. Finally, regarding PUFA, Control showed a high content of linoleic acid (18:2n6), followed by DHA (22:6n3) and EPA (20:5n3). A similar trend was observed in Group A and Group B larvae. Linoleic acid content did not present significant differences among the experimental groups whilst Group A fish were characterized by

significant ($p<0.05$) lower EPA content respect to Control fish, while no significant differences were detected between these groups in terms of DHA content. Differently, both DHA and EPA contents of Group B fish were significantly ($p<0.05$) lower than those of Control fish.

Figure 4 reports the FAs composition (as mg/g dry weight) of zebrafish larvae collected at 21 dps. As concerns SFA content, no significant differences ($p>0.05$) were observed between Group A (40.8±1.7 mg/g) and Control (43.0±4.5 mg/g), while Group B showed a significant ($p<0.05$) higher content compared to the other groups (62.6±2.2 mg/g). Considering MUFA content, no significant differences ($p>0.05$) were detected between Control (40.8±3.5 mg/g) and Group B (45.0±2.0 mg/g), while the content of Group A (30.0±1.3 mg/g) was significantly ($p<0.05$) lower than the other experimental groups. Differently, both Group A (32.0±1.5 mg/g) and Group B (32.1±1.4 mg/g) presented a significant lower ($p<0.05$) PUFA content than Control (37.8±2.4 mg/g). Regarding n3 content, no significant differences ($p>0.05$) were detected between Group A (20.0±1.4 mg/g) and Control (21.4±1.9 mg/g), while the content of Group B (14.9±1.1 mg/g) was significantly ($p<0.05$) lower than that of the Control. In particular, as showed in Table 4, no significant differences ($p>0.05$) were detected between Control and Group A considering DHA and EPA contents whilst they were significantly ($p<0.05$) lower in Group B (DHA: 13.0±0.9 mg/g; EPA: 1.4±0.1 mg/g) compared to Control (DHA: 16.7±1.5 mg/g; EPA: 3.9±0.3 mg/g). No significant differences ($p>0.05$) were evident between Control and Group B in n6 (Control: 16.4±1.4 mg/g, Group B: 17.2±0.8 mg/g) and n9 (Control: 34.6±3.5 mg/g, Group B: 38.1±1.9 mg/g) contents. Conversely, Group A was significantly ($p<0.05$) lower than Control considering both n6 (12.0±0.5 mg/g) and n9 (25.9±1.3 mg/g) contents. Finally, regarding n6/n3, Control ratio (0.8±0.1) was significantly ($p<0.05$) higher than Group A (0.6±0.1), but significantly ($p<0.05$) lower respect to Group B (1±0.1).

Histology

Histological analyses were performed in order to detect possible inflammatory events in the intestine and to evaluate lipid accumulation or steatosis in the liver. No inflammatory events were observed in the intestine of all the analyzed samples at each sampling time (Fig. 5,A). Indeed, the morphology of mucosa appeared unaltered and characterized by the absence of appreciable inflammatory influx in all groups of zebrafish. On the contrary, differences in liver lipid accumulation were observed among the experimental groups (Fig. 5,B). At 7 dps, a consistent lipid accumulation was evident in Group B liver, but not in those of Control and Group A. Lipid vacuoles were still present in Group B livers at 14 dps, but not in those of Group A. At this sampling time, also Control showed the incidence of lipid accumulation. At 21 dps the presence of lipid vacuoles in the liver parenchima was comparable in Group A and Control, while Group B showed the highest accumulation (Fig. 5,B g,h,i).

Real-time PCR results

Real-time PCR analyses were performed on genes involved in fish growth (*igf1*, *igf2a* and *mstnb*), stress response (*hsp70* and *nr3c1*), enzymatic hydrolysis of chitin (*chia.1*, *chia.2*, *chia.3*, *chia.4*, *chia.5* and *chia.6*), long-chain polyunsaturated fatty acids biosynthesis (*elovl2, elovl5* and *fads2*) and immune response (*tnfa* and *il6*).

Growth factors. As reported in Fig. 6a and Fig. 6b, *igf1* and *igf2a* gene expression evidenced similar patterns during the experimental period. For both *igf*s, no significant differences ($p>0.05$) were observed between Group A and Control at each sampling time, with the exception of *igf1* gene expression at 14 dps that was significantly ($p<0.05$) higher in Group A respect to Control. Conversely, Group B was significantly ($p<0.05$) higher than Control at each sampling time, except considering *igf2a* gene expression at 7 dps. A similar pattern was observed for *mstnb* gene expression (Fig. 6c); at 7 dps, Group A showed significantly ($p<0.05$) lower values than Control, while no significant differences ($p>0.05$) were evident between these groups at 14 and 21 dps. On the contrary, Group B showed a significantly ($p<0.05$) higher *mstnb* gene expression respect to Control at 14 and 21 dps.

Stress response. Considering hsp70.1 gene expression (Fig. 7a), no significant differences ($p>0.05$) were observed among the experimental groups at 7 dps. Both Group A and Group B showed significantly ($p<0.05$) higher levels than Control at 14 dps, while at 21 dps, an opposite trend was observed. Regarding nr3c1 gene expression (Fig. 7b), no significant differences ($p>0.05$) were detected among the experimental groups at 14 and 21 dps, while at 7 dps, both Group A and Group B exhibited a significantly ($p<0.05$) lower nr3c1 gene expression compared to Control.

Chitinases. Regarding *chia.1* gene expression (Fig. 8a), no significant differences ($p>0.05$) were detected among experimental groups at 14 dps. Group A did not show significant ($p>0.05$) differences compared to Control at 7 dps, but was significantly ($p<0.05$) lower at 21 dps. Conversely, Group B showed a significantly ($p<0.05$) higher *chia.1* gene expression respect to Control and Group A both at 7 and 21 dps.

As concerns *chia.2* gene expression (Fig. 8b), no significant differences ($p>0.05$) were observed among Control and the treated groups at 14 dps. At 7 dps, no significant differences ($p>0.05$) were evident between Group A and Group B, while Group B resulted significantly ($p<0.05$) higher than Control. At 21 dps, Group A was characterized by a significantly ($p<0.05$) lower *chia.2* gene expression than Control, while Group B showed an opposite trend.

Expression of *chia.3* (Fig. 8c) at 7 dps did not show significant differences ($p>0.05$) among the experimental groups. At 14 dps no significant differences ($p>0.05$) were observed between Group A and Control, while Group B presented

a significantly ($p<0.05$) lower *chia.3* gene expression than Control. At 21 dps, instead, only Group A was significantly lower ($p<0.05$) than Control.

With regard to *chia.4* gene expression (Fig. 8d), no significant differences ($p>0.05$) were detected among the experimental groups at 7 and 14 dps. Both Group A and Group B reached a significantly ($p<0.05$) higher gene expression than Control at 21 dps. Finally, both *chia.5* and *chia.6* gene expression (Fig. 8e,d), at 7 dps, were significantly ($p<0.05$) higher in Group A with respect to Control, while no significant differences ($p>0.05$) were detected between Control and Group B. At 14 dps, significant differences ($p<0.05$) were evident among the experimental groups, with Control higher than Group A, but lower than Group B. At 21 dps, Group A and Group B were characterized by a significantly ($p<0.05$) lower *chia.5* gene expression with respect to Control, while no significant ($p>0.05$) differences were observed among the experimental groups considering *chia.6* gene expression.

Lipid metabolism. Regarding elovl2 gene expression (Fig. 9a), Group A and Control did not show significant differences ($p>0.05$) at all sampling times. Conversely, Group B was characterized by a significantly ($p<0.05$) higher elovl2 gene expression than Control and Group A at 7 and 21 dps. No significant differences ($p>0.05$) were observed among the treated groups and Control at each sampling times considering elovl5 gene expression (Fig.9b). As showed in Figure 9c, fads2 gene expression was not characterized by significant differences ($p>0.05$) among the experimental groups at 7 and 14 dps. Finally, at 21 dps, Group A showed significantly ($p<0.05$) lower values than Control, which did not show significant differences ($p>0.05$) compared to Group B.

Immune response. Regarding *tnfa* gene expression (Fig. 10a), Group A did not show significant differences ($p>0.05$) with respect to Control at all sampling times. Conversely, Group B was significantly ($p<0.05$) higher than Control and Group A at 7 and 21 dps. As concerns *il6* gene expression (Fig. 10b), no significant differences ($p>0.05$) were detected among the experimental groups at 14 dps. At 7 dps, both Group A and Group B were significantly ($p<0.05$) higher than Control, while, at 21 dps, only Group B was characterized by a significantly ($p<0.05$) higher *il6* gene expression with respect to Control.

DISCUSSION

The desirable goal of the aquaculture industry is to replace fish meal and fish oil with more sustainable ingredients.[32] However, alternatives must ensure economic feasibility and a proper fish growth and health by providing adequate levels of nutrients.[4] Insects can exemplify good candidates for a more sustainable aquaculture, representing a valid alternative to fish meal and fish oil. However, their application in aquafeeds still needs to be explored.[11] Many studies have been carried out in the last years on the inclusion of insects in aquafeed formulation;

however, results are still controversial and further research is necessary. For example, replacing up to 25 or 50% of fish meal with mealworm, *Tenebrio molitor* (Coleoptera, Tenebrionidae) had no adverse effects on weight gain in *Sparus aurata*, but fish fed diet with 50% insect substitution showed a growth reduction and less feed conversion ratio compared to control.[33] Similar results were obtained in African catfish (*Clarias gariepinus*), with a replacement of 25% of fish meal by variegated grasshopper, *Zonocerus variegatus* (Orthoptera, Pyrgomorphidae), that improved both growth rate and nutrient utilization. Conversely, when the inclusion of *Z. variegatus* was increased above 50%, growth of *C. gariepinus* was negatively influenced.[34]

One of the limiting factors of including insects in aquafeeds is their fatty acid profile that does not always match the nutritional requirements of fish. It is known that in insects, the quantity and quality of fatty acids are species specific, vary with the developmental stage and can be modified by the growth substrate.[35,36] Among several insect species, BSF is one of the most promising because it shows an essential amino-acid profile similar to that of fish meal.[9] In addition, its lipid profile can be manipulated by changing the composition of the growth substrate.[35] The present study investigated for the first time the formulation of new test diets including different substitution levels of fish meal and fish oil with full-fat BSF meal, during zebrafish larviculture. Zebrafish is a widely used model organism but its dietary preferences and its nutritional requirements are mostly unknown.[20] Results obtained from the present study may be used to generalize how several biological processes occur in related species, especially in farmed fish, improving our understanding on the mechanisms involved in fish nutrition and growth.[37]

In order to gain a deeper knowledge about the effects of the inclusion of BSF meal in aquafeeds a comprehensive multidisciplinary approach was applied.

Generally, all the tested experimental diets did not show significant negative effects on larval survival. Indeed, the experimental groups showed comparable survival rates, in accordance with previous studies on various fish species.[18,19,38,39] Growth (standard length and mean dry weight) was also similar among the groups up to 14 dps; however, even if the three diets were isoenergetic, at 21 dps, Group B larvae were significantly bigger (standard length and mean dry weight) respect to both Control and Group A larvae. All biometric data were fully supported by the molecular results about growth factors (*igfs* and *mstn*) and were in accord with previous studies on other fish species such as rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*) and blue tilapia (*Oreochromis aureus*).[40,41]

One of the main problems of the use of insect meal in aquafeed is related to its FA profile which in turn affects the fish quality as reported by St-Hilaire et al. (2007).[35] This could be problematic for consumers who desire fish with high concentrations of long-chain unsaturated fatty acids.

In the present study, the three different diets showed a similar total lipid content but a different FA composition. In particular, diets with higher inclusions of BSF meal showed an increase in SFA and a decrease in PUFA.

However, regarding PUFAs, a reduction in these severe differences was observed in zebrafish larvae fed the different diets. A reasonable explanation of this result is the ability of many freshwater species, including zebrafish, to convert shorter-chain precursors in highly unsaturated FAs through the activation of specific elongase and desaturase (*elovl2*, *elovl5* and *fad*).[42]

As regards SFA, differences between diets and larval composition were less evident. In fact, a significant increase in SFA larval content was observed only with the highest BSF meal inclusion (Group B). Group B larvae where particularly rich in lauric acid (C12). This result is in line with other studies[22,43] reporting that lauric acid, a medium chain fatty acid particularly abundant in insects (mainly in *H. illucens*), is usually preferentially utilized as energy source and efficiently absorbed, digested and β-oxidised if provided in adequate amounts through the diet.[44]

Even if the three offered diets were isolipidic, larval fish hepatic accumulation was very different. In fact, only group B larvae showed a severe lipid steatosis in the liver.

Since n-3 PUFA are known to limit triglyceride deposition in the liver, steatosis has been reported to be related to a PUFA deficiency, and to high n-6/n-3 ratio.[45,46] In addition, a recent review reports that an increase in the dietary SFA may play an important role in the development of hepatic steatosis,[47] causing liver dysfunction by promoting endoplasmic reticulum stress and apoptosis.[48–50]

As a consequence, the observed lipid steatosis of Group B larvae can be related to the quality rather than the quantity of the dietary fat provided through the three different diets.

In fact, the above mentioned *scenario* was fully represented only in Group B: lower n-3 and higher SFA content and a higher n6/n3 ratio respect to Control and Group A.

Medium-chain fatty acids have also been reported to possess positive properties such as antibacterial and antiviral activity and have demonstrated positive effects improving gut health under inflammatory conditions.[51–53] Insects are rich in chitin which may induce intestinal inflammation and a reduction in fish welfare and growth.[54,55] However, in the present study, no signs of intestinal inflammation were observed, possibly due to the high supplementation of medium-chain fatty acids through the diet. These data were also generally supported by molecular analysis on stress markers that did not show significant variations in their expression during the experiment.

However, it should be pointed out that the present study was only 21 days long and pathological signs could become evident over a longer period of time.

As a consequence, molecular markers can be useful to precociously detect physiological responses. In the present study, genes involved in the immune response (*il6* and *tnfa*) were highly expressed in Group B compared to Control and Group A. This result was not obvious and can possibly be related to an upcoming intestinal inflammation or to the already evident hepatic steatosis.

Finally, different fish species may have different chitinolytic activity. Some species, like turbot (*Scophthalmus maximus*), do not have any chitinolytic activity during the early life stages,[15] while others, like cobia (*Rachycentron canadum*), showed very high endochitinolytic activity.[56] Generally, the analysis of the six zebrafish chitinases in larvae fed different diets showed that chitinase gene expression was not totally dependent on the amount of chitin provided through the diet. This result was not obvious and a possible involvement of intestinal bacteria with chitinolytic activity should be considered in future studies.

CONCLUSION

The present study tested for the first time the partial dietary inclusion of *Hermetia illucens* meal in zebrafish larval diets and the physiological responses of the larvae. Generally, results are promising but a 50% BSF meal dietary inclusion affected both lipid composition and accumulation of the larvae. Further studies are needed to better understand the physiological responses of fish, including very low BSF-meal substitutions which may play a immunomodulatory role.

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Table 1. Ingredient composition, proximate analysis and gross energy content of the test diets.

	Control	Group A	Group B
Ingredients (g/kg)			
Fish meal, Chile, super prime	420	315	210
Peas, protein concentrate	55	78	100
Hermetia illucens	0	105	210
Wheat, gluten meal	55	78	100
Wheat flour	290	268	255
Fish oil	70	40	28
Palm oil	70	75	56
Min. & Vit. Supplement §	20	20	20
Binder	20	20	20
L-methionine	0	1	1
Proximate composition (%)			
Dry matter	4.2±0.03	5.5±0.18	5.3±0.42
Crude protein	40.0±0.47	40.2±0.39	41.1±0.10
Crude lipid	18.6±0.14	17.7±0.20	17.0±0.13
Ash	14.2±0.23	14.1±0.31	12.2±0.59
N-free extractive (NFE)	23.0±0.31	22.5±0.60	24.4±0.99
Gross Energy (MJ/kg)	22.1±0.11	22.3±0.03	21.3±0.06

§Composition of mineral mix (g/kg diet): Ca HPO_4 *$2H_2O$, 27.5; K_2HPO_4, 19.0; NaCl, 6.1; MgO, 2.0; $FeCO_3$, 1.75; KI, 0.15; ZnO, 0.11; MnO, 0.07; $CuSO_4$, 0.02; sodium selenite, 0.002. Composition of vitamin mix (mg/kg diet): thiamin HCl, 40; riboflavin, 40; pyridoxine HCl, 40; cyanocobalamine, 0.2; niacin, 300; calcium panthothenate, 100; folic acid, 5; biotin, 3; choline chloride, 5000; myo-inositol, 1000; ascorbic acid, 2000; a-tocopheryl acetate, 250; menadione, 90; vit. A retinyl palmitate, 40,000 IU/kg diet; vit. D_3 cholecalciferol. 2500 IU/kg diet.

Table 2. Primer sequences and ZFID used in the present study.

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')	ZFIN ID
igf1	5'-GGCAAATCTCCACGATCTCTAC-3'	5'-CGGTTTCTCTTGTCTCTCTCAG-3'	ZDB-GENE-010607-2
igf2a	5'-GAGTCCCATCCATTCTGTTG-3'	5'-GTGGATTGGGGTTTGATGTG-3'	ZDB-GENE-991111-3
mstnb	5'-GGACTGGACTGCGATGAG-3'	5'-GATGGGTGTGGGGATACTTC-3'	ZDB-GENE-990415-165
hsp70.1	5'-TGTTCAGTTCTCTGCCGTTG-3'	5'-AAAGCACTGAGGGACGCTAA-3'	ZDB-GENE-990415-91
nr3c1	5'-AGACCTTGGTCCCCTTCACT-3'	5'-CGCCTTTAATCATGGGAGAA-3'	ZDB-GENE-050522-503
chia.1	5'-ACTGGGCGGAGCCTCAGTGT-3'	5'-GGGCTTGGGTGGGAAACCCAG-3'	ZDB-GENE-040426-1994
chia.2	5'-GGTGCTCTGCCACCTTGCCTT-3'	5'-GGCATGGTTGATCATGGCGAAAGC-3'	ZDB-GENE-040426-2014
chia.3	5'-TCGACCCTTACCTTTGCACACACCT-3'	5'-ACACCATGATGGAGAACTGTGCCGA-3'	ZDB-GENE-040426-2891
chia.4	5'-TGGACACCTCCACACGCTGC-3'	5'-ATGCCCACTAATCCGCCCGC-3'	ZDB-GENE-030131-9279
chia.5	5'-CCACGGCTCACAGGACAACATCA-3'	5'-GTCCGCAGACGACAGGCGAA-3'	ZDB-GENE-071004-113
chia.6	5'-TCCACGGCTCATGGGAGAGTGTC-3'	5'-AGCGCCCTGATCTCGCCAGT-3'	ZDB-GENE-030131-1140
elovl2	5'-CACTGGACGAAGTTGGTGAA-3'	5'-GTTGAGGACACACCACCAGA-3'	ZDB-GENE-060421-5612
elovl5	5'-TGGATGGGACCGAAATACAT-3'	5'-GTCTCCTCCACTGTGGGTGT-3'	ZDB-GENE-040407-2
fads2	5'-CATCACGCTAAACCCAACA-3'	5'-GGGAGGACCAATGAAGAAGA-3'	ZDB-GENE-011212-1
tnfa	5'-TTGTGGTGGGGTTTGATG-3'	5'-TTGGGGCATTTTATTTTGTAAG-3'	ZDB-GENE-050317-1
il6	5'-CTGGAGGCCATAAACAGCCA-3'	5'-TGCGAGTCCATGCGGATTTA-3'	ZDB-GENE-120509-1
arpc1a	5'-CTGAACATCTCGCCCTTCTC-3'	5'-TAGCCGATCTGCAGACACAC-3'	ZDB-GENE-040116-1
rpl13	5'-TCTGGAGGACTGTAAGAGGTATGC-3'	5'-AGACGCACAATCTTGAGAGCAG-3'	ZDB-GENE-031007-1

Table 3. Fatty acid composition (% FAMEs) of experimental diets. Means within rows bearing different letters differ significantly ($p<0.05$).

	Control	Group A	Group B
10:0	n.d.	0.36 ± 0.03^{a}	0.74 ± 0.04^{a}
12:0	0.14 ± 0.05^{a}	6.49 ± 0.68^{b}	13.05 ± 1.37^{c}
13:0	0.05 ± 0.02^{a}	0.04 ± 0.05^{a}	0.07 ± 0.04^{a}
14:0	4.33 ± 0.16^{a}	4.02 ± 0.31^{a}	5.68 ± 1.05^{b}
15:0	0.70 ± 0.09^{a}	0.43 ± 0.08^{a}	0.54 ± 0.26^{a}
16:0	15.07 ± 1.63^{b}	24.11 ± 2.81^{a}	21.62 ± 1.21^{a}
16:1n9	0.18 ± 0.04^{a}	0.13 ± 0.05^{a}	0.18 ± 0.02^{a}
16:1n7	5.30 ± 0.36^{a}	3.88 ± 0.91^{b}	5.25 ± 0.84^{a}
17:0	0.77 ± 0.11^{a}	0.48 ± 0.04^{a}	0.59 ± 0.13^{a}
18:0	4.89 ± 0.74^{a}	5.44 ± 1.11^{a}	5.08 ± 0.23^{a}
18:1n9	13.24 ± 0.55^{a}	27.85 ± 1.65^{b}	24.97 ± 1.76^{b}
18:1n7	2.14 ± 0.38^{a}	0.99 ± 0.16^{b}	0.98 ± 0.31^{b}
18:2n6	11.24 ± 1.53^{a}	8.95 ± 1.46^{ab}	8.03 ± 1.49^{b}
18:3n6	0.19 ± 0.03^{a}	0.07 ± 0.04^{ab}	0.05 ± 0.06^{b}
18:3n3	2.44 ± 0.13^{a}	1.13 ± 0.23^{b}	0.95 ± 0.35^{b}
20:0	0.36 ± 0.07^{a}	0.42 ± 0.14^{a}	0.45 ± 0.07^{a}
20:1n9	1.45 ± 0.35^{a}	0.86 ± 0.22^{a}	0.94 ± 0.20^{a}
20:2n6	0.28 ± 0.01^{a}	0.10 ± 0.0^{b}	0.08 ± 0.03^{c}
20:3n6	0.20 ± 0.04^{a}	0.07 ± 0.08^{b}	0.05 ± 0.03^{b}
21:0	0.07 ± 0.05^{a}	0.03 ± 0.03^{a}	0.02 ± 0.02^{a}
20:4n6	1.21 ± 0.14^{b}	0.49 ± 0.13^{a}	0.41 ± 0.06^{a}
20:3n3	0.22 ± 0.07^{a}	0.10 ± 0.14^{a}	0.07 ± 0.04^{a}
20:5n3	11.34 ± 0.24^{a}	4.24 ± 0.49^{b}	3.12 ± 0.74^{b}
22:0	0.26 ± 0.03^{a}	0.27 ± 0.01^{a}	0.36 ± 0.07^{a}
22:1n9	0.92 ± 0.13^{a}	0.39 ± 0.05^{b}	0.35 ± 0.08^{b}
24:0	0.01 ± 0.01^{a}	0.05 ± 0.02^{a}	0.04 ± 0.05^{a}
22:6n3	22.23 ± 0.89^{c}	8.24 ± 0.36^{b}	5.97 ± 0.72^{a}
24:1n9	0.77 ± 0.38^{a}	0.37 ± 0.08^{a}	0.38 ± 0.08^{a}

Table 4. Fatty acid composition (as mg/g dw) of zebrafish larvae collected at 21 dps. Means within rows bearing different letters are significantly different ($p<0.05$).

	Control	Group A	Group B
10:0	n.d.^{a}	0.12±0.03^{a}	0.17±0.13^{a}
12:0	0.29±0.04^{a}	3.63±0.97^{b}	12.31±2.06^{c}
13:0	0.07±0.01^{ab}	0.04±0.00^{a}	0.11±0.04^{b}
14:0	3.50±0.32^{a}	3.47±0.53^{a}	8.24±1.92^{b}
15:0	0.81±0.02^{ab}	0.60±0.02^{a}	1.10±0.32^{b}
16:0	24.47±1.31^{a}	20.20±2.66^{a}	26.26±5.81^{a}
16:1n9	0.89±0.21^{ab}	0.63±0.09^{a}	1.35±0.32^{b}
16:1n7	3.21±0.62^{ab}	2.08±0.55^{a}	3.94±0.84^{b}
17:0	0.95±0.03^{ab}	0.75±0.06^{a}	1.23±0.29^{b}
18:0	12.47±0.56^{a}	11.66±0.35^{a}	12.73±1.58^{a}
18:1n9	32.05±0.71^{a}	24.52±5.35^{a}	35.96±5.79^{a}
18:1n7	3.06±0.04^{b}	2.09±0.36^{a}	3.01±0.48^{b}
18:2n6	14.00±0.52^{a}	9.71±2.29^{a}	14.05±2.33^{a}
18:3n6	0.20±0.00^{b}	0.11±0.02^{a}	0.19±0.03^{b}
18:3n3	0.48±0.03^{b}	0.21±0.05^{a}	0.31±0.07^{a}
20:0	0.48±0.00^{b}	0.35±0.05^{a}	0.41±0.04^{ab}
20:1n9	1.38±0.21^{b}	0.56±0.09^{a}	0.61±0.10^{a}
20:2n6	0.88±0.08^{a}	0.65±0.17^{a}	0.83±0.13^{a}
20:3n6	0.88±0.01^{a}	0.98±0.16^{a}	1.54±0.17^{b}
20:4n6	0.45±0.01^{a}	0.54±0.02^{b}	0.60±0.05^{b}
20:3n3	0.37±0.02^{b}	0.20±0.05^{a}	0.23±0.03^{a}
20:5n3	3.86±0.32^{b}	1.78±0.28^{a}	1.40±0.14^{a}
22:1n9	0.25±0.00^{b}	0.15±0.02^{a}	0.15±0.01^{a}
22:6n3	16.73±1.47^{b}	17.86±1.63^{b}	12.97±0.85^{a}

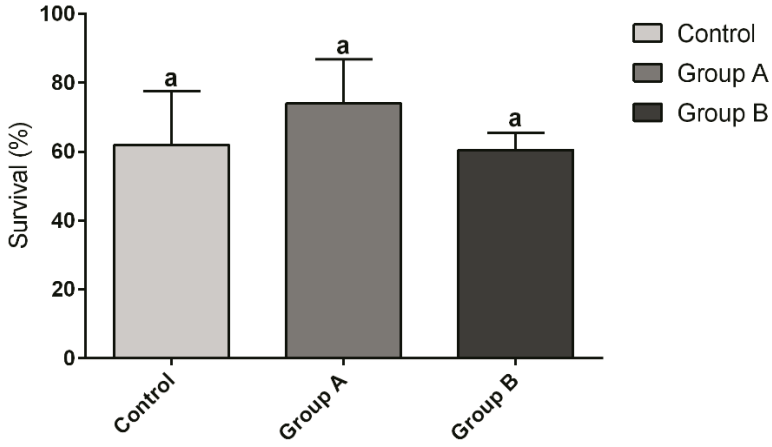

Figure 1. Survival (in %) of zebrafish larvae fed diet based on fish meal (Control) and diets with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

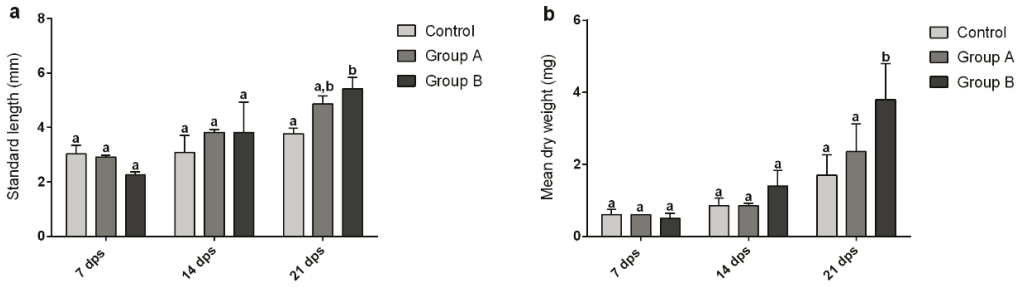

Figure 2. (a) Standard length (mm) and (b) mean dry weight (mg) of zebrafish larvae fed diet based on fish meal (Control) and diets with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal. Mean dry weight was referred to pools of 10 animals for each group. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p<0.05$). Values are presented as mean ± SD (n = 3).

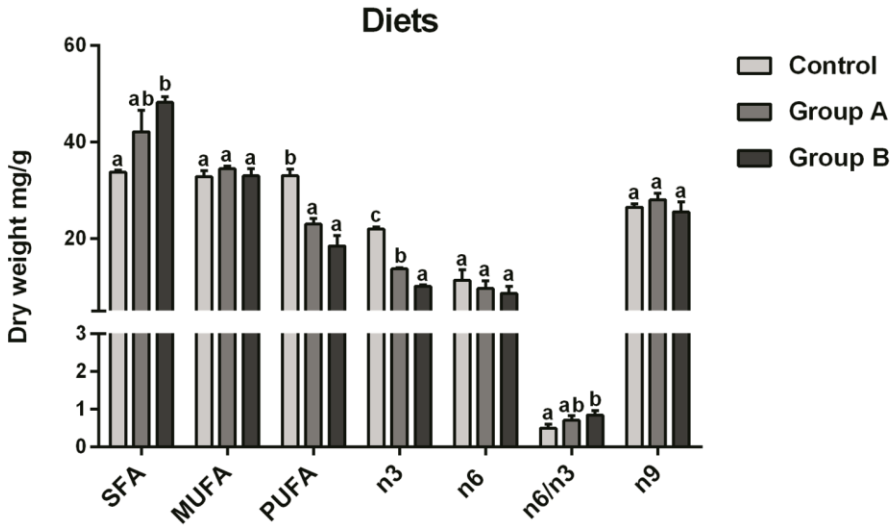

Figure 3. Content of saturated (SFA), mono-unsaturated (MUFA) and poly-unsaturated (PUFA) fatty acids in the experimental diets, contribution of n3, n6 and n9 fatty acids to lipid profiles (as mg/g dry weight) and n6/n3 ratio. Different letters indicate statistically significant differences among experimental groups compared within the same fatty acid class ($p<0.05$). Values are presented as mean ± SD ($n = 3$). Control diet was based on fish meal, while Group A and Group B diets were characterized by 25 or 50% replacement of fish meal with BSF meal, respectively.

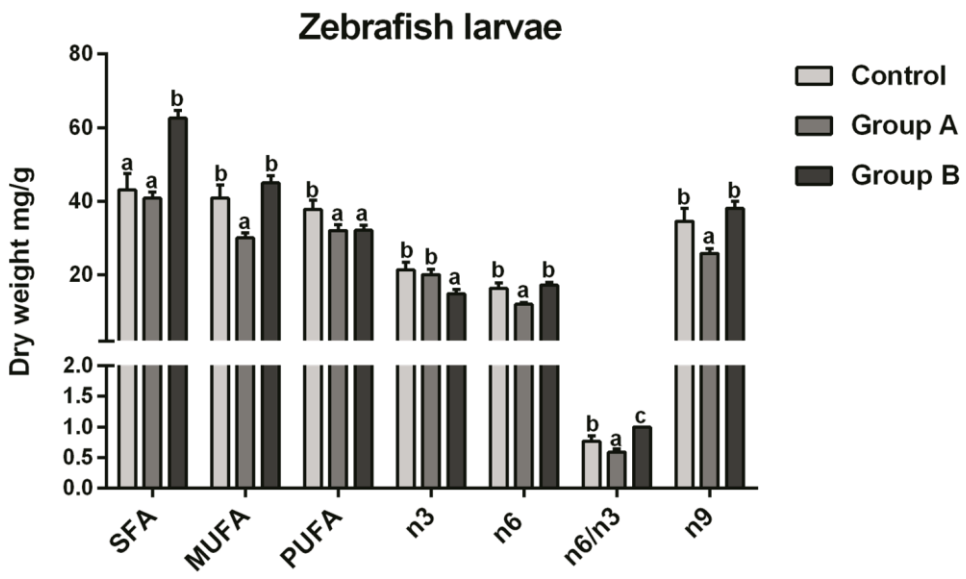

Figure 4. Content of saturated (SFA), mono-unsaturated (MUFA) and poly-unsaturated (PUFA) fatty acids in zebrafish larvae, contribution of n3, n6 and n9 fatty acids to lipid profiles (as mg/g dry weight) and n6/n3 ratio. Different letters indicate statistically significant differences among experimental groups compared within the same fatty acid class ($p<0.05$). Values are presented as mean ± SD ($n = 3$). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

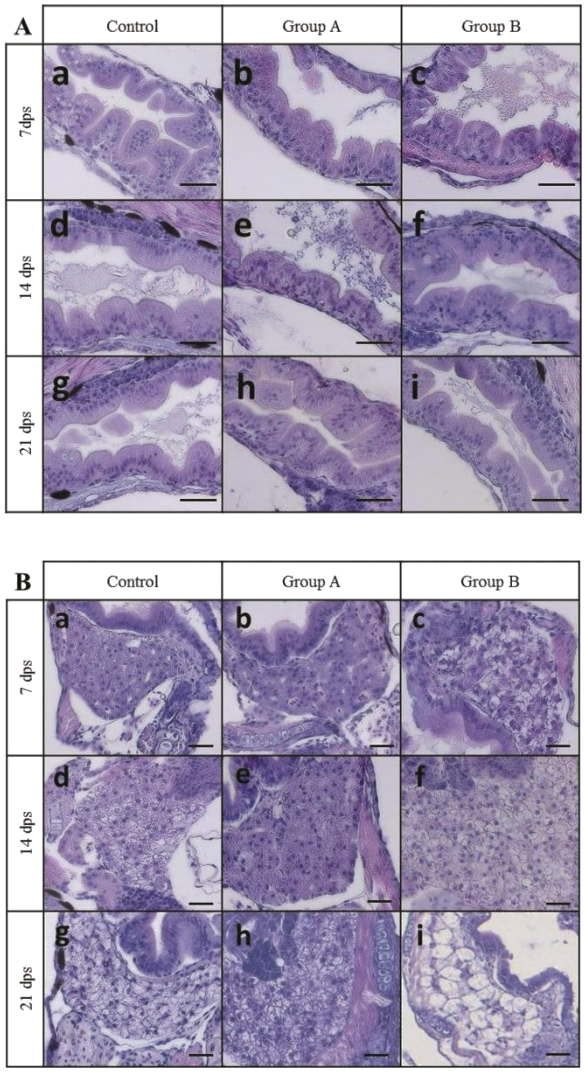

Figure 5. Example of histomorphology (at 7,14 and 21 dps) of zebrafish: (A) intestine Control (a,d,g), Group A (b,e,h), Group B (c,f,i); (B) liver Control (a,d,g), Group A (b,e,h), Group B (c,f,i). Scale bars: 25 μm. Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

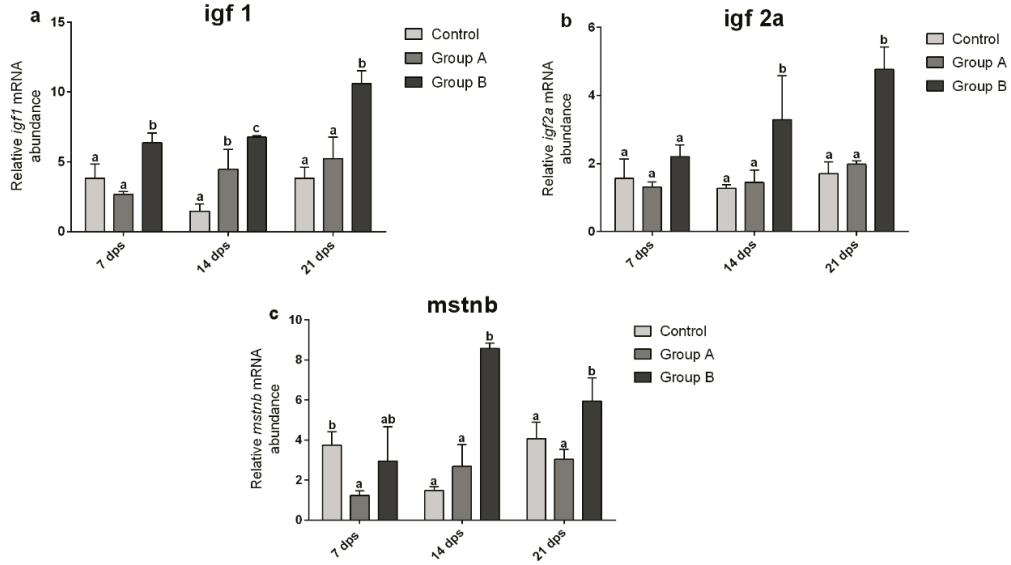

Figure 6. Relative mRNA levels of genes involved in growth analyzed in zebrafish larvae from the three experimental groups at 7, 14 and 21 dps. (a) *igf1*, (b) *igf2a*, (c) *mstnb*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p<0.05$). Values are presented as mean ± SD ($n = 3$). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

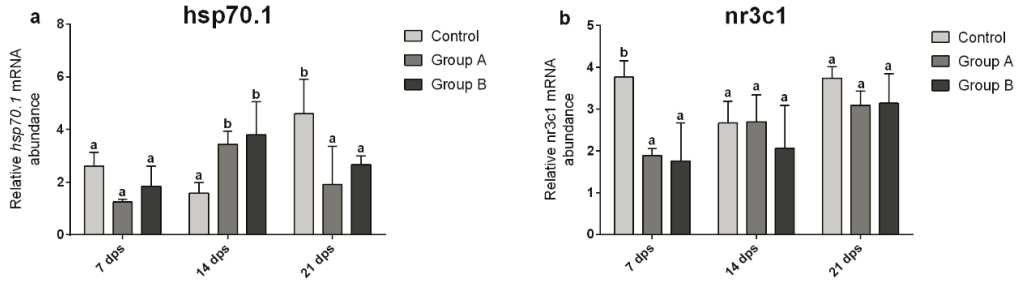

Figure 7. Relative mRNA levels of genes involved in stress response analyzed in zebrafish larvae from the three experimental groups at 7, 14 and 21 dps. (a) *hsp70.1*, (b) *nr3c1*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p<0.05$). Values are presented as mean ± SD (n = 3). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

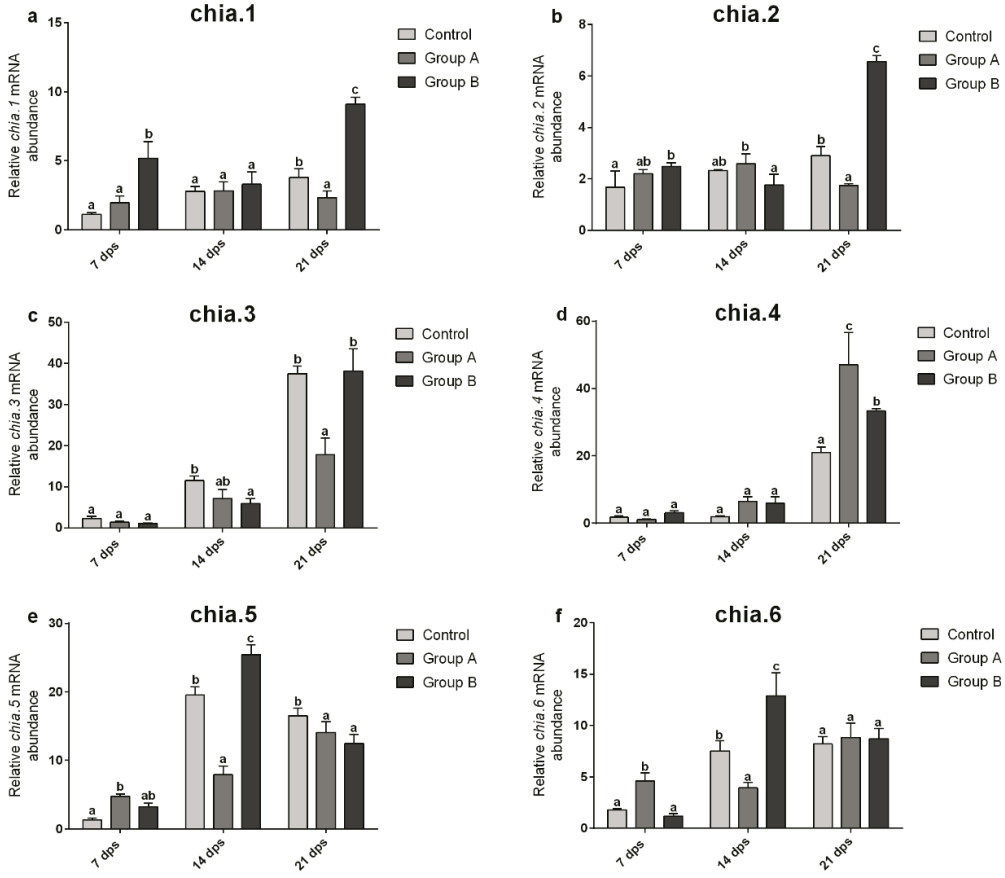

Figure 8. Relative mRNA levels of genes involved in enzymatic hydrolysis of chitin analyzed in zebrafish larvae from the three experimental groups at 7, 14 and 21 dps. (a) *chia.1*, (b) *chia.2*, (c) *chia.3*, (d) *chia.4*, (e) *chia.5*, (f) *chia.6*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p<0.05$). Values are presented as mean ± SD (n = 3). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

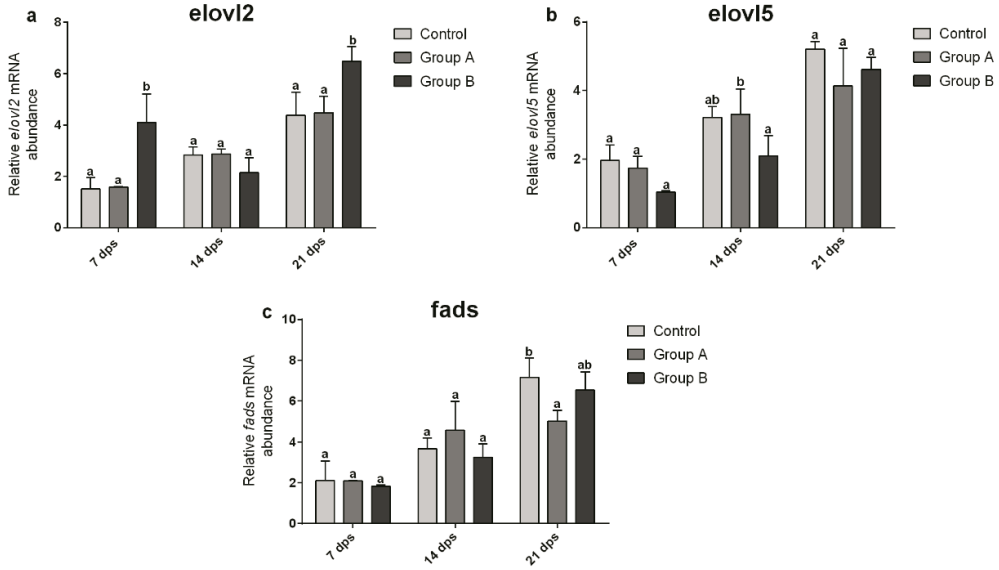

Figure 9. Relative mRNA levels of genes involved in lipid metabolism and in the long-chain polyunsaturated fatty acids biosynthesis analyzed in zebrafish larvae from the three experimental groups at 7, 14 and 21 dps. (a) *elovl2*, (b) *elovl5*, (c) *fads2*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p<0.05$). Values are presented as mean ± SD (n = 3). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

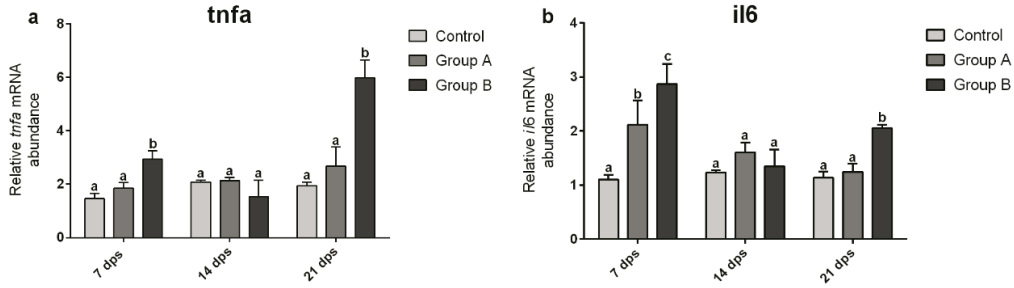

Figure 10. Relative mRNA levels of genes involved in immune response analyzed in zebrafish larvae from the three experimental groups at 7, 14 and 21 dps. (a) *tnfa*, (b) *il6*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p<0.05$). Values are presented as mean ± SD ($n = 3$). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

Paper 7

animals

MDPI

Article

Effects of Graded Dietary Inclusion Level of Full-Fat *Hermetia illucens* Prepupae Meal in Practical Diets for Rainbow Trout (*Oncorhynchus mykiss*)

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Simple Summary: The sustainability of fish production is mainly driven by the protein source used in aquafeeds. In conventional fish feed, protein sources are mostly vegetable ingredients and fishmeal. The present study explored the potential use of full-fat *Hermetia illucens* prepupae meal (H) replacing 0% (H0), 25% (H25), and 50% (H50) conventional ingredients in practical diets for rainbow trout. No significant differences in growth were observed in all experimental groups, while in fish fed the H50 diet both hepatic and intestinal alterations were detected. In addition, in the same fish group, genes related to stress and immune-response were significantly up-regulated. The results obtained so far highlighted an overall physiological adaptation of fish to the dietary manipulation, suggesting an adverse effect of full-fat H at the highest inclusion level.

Abstract: This study investigated the effects of dietary inclusion levels of full-fat *Hermetia illucens* prepupae meal (H) on growth and gastrointestinal integrity in rainbow trout (*Oncorhynchus mykiss*). A 98-day study was conducted using triplicate groups of trout (initial body weight, 137 ± 10.5 g) kept in 1-m^3 tanks in a flow-through well water system. Three dietary treatments were prepared: one based on fishmeal and purified protein-rich vegetable ingredients (H0), and two experimental diets including graded levels of H meal (25% and 50%, referred to as H25 and H50, respectively). At the end of the feeding trial, no differences were observed in growth performance and plasma metabolite levels, with the biometric data confirmed by the liver expression of the genes involved in somatic growth regulation (*igf1* and *mstn1a*). In the H50 group, a three-fold up regulation of liver *hsp70* was observed. An activation of the stress/immune response (*il-10*, *tnf-α*, and *tlr-5*) was observed in medium intestine in the H25 and H50 groups ($p < 0.05$) together with a villi length reduction detected through histological analyses. Liver histology and Fourier Transform Infrared Imaging (FTIRI) spectroscopy highlighted an increase in lipid deposition. These findings suggest that caution should be taken into account when 50% replacement of conventional ingredients with H is selected.

Keywords: black soldier fly; feed formulation; alternative proteins; growth metrics; FTIRI spectroscopy; gastrointestinal health; plasma metabolite

Effects of graded dietary inclusion level of full-fat *Hermetia illucens* prepupae meal in practical diets for rainbow trout (*Oncorhynchus mykiss*)

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§These two authors equally contributed to the study

Simple Summary: The sustainability of fish production is mainly driven by the protein source used in aquafeeds. In conventional fish feed protein sources are mostly vegetable ingredients and fishmeal. The present study explored the potential use of full-fat Hermetia illucens prepupae meal (H) replacing 0 (H0), 25% (H25) and 50% (H50) conventional ingredients in practical diets for rainbow trout. No significant differences in growth were observed in all experimental groups, while in fish fed diet H50 both hepatic and intestinal alterations were detected. In addition, in the same fish group, genes related to stress and immune-response were significantly up-regulated. The results obtained so far, highlighted an overall fish physiological adaptation to the dietary manipulation, suggesting an adverse effect of full-fat H at the highest inclusion level.

Abstract: This study investigated the effects of dietary inclusion levels of full-fat *Hermetia illucens* prepupae meal (H) on growth and gastrointestinal integrity in rainbow trout (*Oncorhynchus mykiss*). A 98-day study was conducted using triplicate groups of trout (initial body weight, 137 ± 10.5 g) kept in 1 m^3 tanks in a flow-through well water system. Three dietary treatments were prepared: one based on fishmeal and purified protein-rich vegetable ingredients (H_0) and two experimental diets including graded levels of H meal (H_{25} and H_{50}). At the end

of the feeding trial, no differences were observed in growth performance and plasma metabolite levels, with the biometric data confirmed by the liver expression of the genes involved in somatic growth regulation (*igf1* and *mstn1a*). In H50 group, a three-fold up regulation of liver *hsp70* was observed. An activation of the stress/immune response (*il-10*, *tnf-a* and *tlr*-5) was observed in medium intestine in H25 and H50 groups ($p < 0.05$) together with a villi length reduction detected through histological analyses. Liver histology and Fourier Transform Infrared Imaging (FTIRI) spectroscopy highlighted an increase in lipid deposition. These findings suggest that caution should be taken into account when 50% replacement of conventional ingredients with H is selected.

Keywords: Black soldier fly, feed formulation, alternative proteins, growth metrics, FTIRI spectroscopy, gastrointestinal health, plasma metabolite.

1. Introduction

One of the most critical issues that threatens the sustainability and further growth of intensive aquaculture of carnivorous species is its dependency on fishmeal (FM) and fish oil (FO) in aquafeed [1]. Thus, alternative ingredients are needed to promote a sustainable aquaculture production while improving fish growth and health performance [2]. Over the last two decades, research efforts focused on the reduction of the dietary inclusion levels of FM and FO by replacement with plant protein sources and vegetable oils, readily available and cost-effective on the feed market [1]. However, even if plant protein-based diets provided good results in some aquatic species, they presented some disadvantages for fish welfare and are often in direct competition with human nutrition [3,4,5] raising pressures on the search of alternative and nutritional strategies to improve their utilization [3]. Some alternatives include animal feedstuffs, comprising both non-ruminant slaughterhouse by-products (derived from processed animal proteins, PAPs) and insect meals [6,7]. Insect meals show several advantages compared to conventional PAPs (poultry meal, hydrolysed poultry feathers, blood meal), as insects grow and reproduce quickly and easily on low-quality organic products [7], have low ecological footprint [8], high feed conversion efficiency [9] and, together with PAPs, can reasonably foster a circular bio-economy.

In July 2017, the European Commission allowed the use of seven insect species as processed animal protein for aquaculture (Regulation 2017/893/EC, 2017). Although insect nutrient composition is dependent on taxonomic group, rearing substrate and technological process, they usually show high protein content (60–80% dry matter basis) with a well-balanced essential amino acid profile [10], valuable lipid, mineral and vitamin content [11,12]. These nutritional properties allows insects to be considered as a valuable alternative protein source, especially for carnivorous fish species, and several reviews on this topic are now available [11-13].

Among the different insect species considered for possible use in fish feeds [12,13], the dipteran *Hermetia illucens*, also known as black soldier fly (BSF), seems to be the most promising in that high quality standard industrial mass-rearing techniques already exist. The protein and lipid content of *H. illucens* meal (H) is variable depending on the processing technology and growth substrate. Generally, on dry matter basis, the defatted *H. illucens* meal protein and lipid content ranges from 47.2 to 51.8% and from 11.8 to 14.8%, respectively [14,15]; whereas the protein and lipid contents reported for full-fat *H. illucens* meal are 36.2 and 18.0% [11], respectively. A major drawback in using *H. illucens* meal as an ingredient in aquafeeds is its lack of long chain polyunsaturated fatty acids (LC-PUFAs) such as both the eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids. The insect meal is rich in linoleic acid (LA, 18:2n-6), saturated (SFAs) and monounsaturated (MUFA) fatty acids. The poor LC-PUFA content may be reflected in a low EPA and DHA content in the edible portion of fish (fillet), thus affecting the nutritional value for consumers. However, the great plasticity of *H. illucens* lipid composition, recently demonstrated by rearing the larvae on n-3 enriched substrates such as fish offal [16] or seaweeds [17], contributes to continuing interest in this species.

Despite the nutritional value of *H. illucens* meal (H), its successful inclusion level in aquafeeds depends on the fish species and the characteristics of insect derivatives [13]. Newton et al. [18] reported similar weight gain for channel catfish (*Ictalurus punctatus*) fingerlings fed diets containing up to 30% of full-fat *H. illucens* prepupae meal. St-Hilaire et al. [19] did not find any negative effects on rainbow trout (*Oncorhynchus mykiss*) growth performance when a 15% full-fat *H. illucens* prepupae meal was included in the diet. The growth performance of trout fed diets containing 25% and 50% (dry matter basis) of H. illucens meal from prepupae reared on fish by-product enriched substrates, showed results similar to those of fish fed the control FM based diet [20]. Replacing up to 50% of FM with partially defatted-BSF larvae resulted in growth performance, body indices and gut morphology similar in rainbow trout [21].

An additional controversial problem related to the inclusion of insect meal in aquafeeds is the presence of chitin [12]. Chitin was thought to play a role in shaping the gut microbial community [22] and to have positive effects on the innate immune response at moderate inclusion level in the diet (25-50 mg kg-1 chitin) [23]. However, when higher inclusion level are used, the same macromolecule can adversely affect nutrients digestibility and uptake in fish [14,21,24] and negatively affects the intestinal mucosa integrity in amphibians [25].

Adequate nutrition is essential to guarantee fish health and digestive capacity; the morphological changes associated with the digestive system (liver and intestinal tract) are usually taken into account when alternative ingredients are considered in aquafeeds. In this regard, investigation of digestive system function of salmonids have been performed using histological [21,26,27,28], transcriptomic [29], proteomic [30] and molecular [31] approaches. The Fourier Transform

Infrared Imaging (FTIRI) spectroscopy, a fast, new and label-free inexpensive technique, is becoming more widely used. FTIRI allows researchers to obtain important biochemical information on the composition of biological samples through the macromolecule structures identification (lipids, proteins, carbohydrates and nucleic acids) using the same sample at the same time. This method has recently been applied to the macromolecular characterization of trout and zebrafish (*Danio rerio*) intestine [32,33], gilthead seabream (*Sparus aurata*) liver [34] and clownfish (*Amphiprion ocellaris*) liver and intestine [35]. In the aforementioned studies, FTIRI highlighted that zebrafish fed insect meal-based diets resulted in a change of the intestinal mucosa macromolecular composition, showing an increase in the relative quantity of protein and fatty acid with longer alkyl chains [33]. On the other hand, the liver macromolecular composition of clownfish fed increasing levels of defatted *H. illucens* larvae meal [35], resulted in a lipid and protein decrease and an increase in glycogen. To the best of our knowledge, no similar information is available yet on trout fed diets including full-fat insect meal.

Coupled with the evaluation of gastrointestinal morphology and/or macromolecular composition, the relationship between dietary changes and metabolic profile, cytokine production [25], potential induction of inflammation [36,37] and stress biomarkers, may represent an useful tool for the overall fish welfare status evaluation.

The freshwater species of most interest to Italian aquaculture is rainbow trout (*Oncorhynchus mykiss*), with a volume of around 35.000 tons, which corresponds to 24.5% of the total national production [38]. In the present study, the effect of graded inclusion level of full-fat Hermetia illucens meal in a practical diet for this species was evaluated by assessing some growth indexes, plasma metabolites, gastrointestinal health and stress biomarkers, using a multidisciplinary approach. The dietary inclusion levels of full-fat H meal were based on previous studies of insect meal [19,20] or other alternative vegetable protein sources [39-42] substitution. The hypothesis considered whether or not the full-fat H meal was a suitable alternative protein ingredient in a practical diet for rainbow trout without impairing fish welfare.

2. Materials and Methods

2.1 Experimental diets

A full-fat *H. illucens* prepupae meal (H) was purchased from the Smart Bugs s.s. (Ponzano Veneto, Italy) company and stored at -20 °C in zip-lock bags until use. No information was provided by the producer on the composition of different vegetable substrate used for larval rearing, as it was considered confidential. Frozen prepupae were grinded with Retsch Centrifugal Grinding Mill ZM 1000 (Retsch GmbH, Germany) and immediately used to prepare experimental diets. A basal diet (Ho) containing fish meal, pea protein concentrate and wheat gluten

meal was formulated to obtain 40 g 100 g-1 crude protein (CP), 18.5 g 100 g-1 ether extract (EE) and 22 MJ kg-1 gross energy (GE), in order to meet the nutrient requirements of rainbow trout [43]. The other two isonitrogenous, isolipidic and isoenergetic experimental diets were prepared by including graded levels of insect meal (H25 and H50) in the Ho formulation. The experimental feeds were prepared at the Department of Agricultural, Food, Environmental and Animal Science (Di4A) of the University of Udine (Udine, Italy). All the ground ingredients (0.5 mm) and fish and vegetable oils were thoroughly blended (Kenwood kMix KMX53 stand Mixer; Kenwood,) for 20 minutes and water was then added to the mixture to attain appropriate consistency for pelleting. Pellets were obtained by using a 4.5 mm die meat grinder and dried at 40 °C for 48-72 h. The obtained diets were subsequently stored in under vacuum bags and kept at −20 °C until used. Diet formulation and proximate composition are shown in Table 1.

Table 1. Ingredients (g kg-1), proximate composition (g 100 g-1) and summary of the major fatty acid classes (as percentage of total FAMEs) of prepupae meal (H) and the experimental diets.

	H	Ho	H25	H50
Ingredients				
Chile prime fish meal[1]		420	315	210
Pea protein concentrate[2]		55	78	100
H meal[3]		-	105	210
Wheat gluten meal[4]		55	78	100
Wheat flour[5]		290	268	255
Fish oil		70	40	28
Palm oil		70	75	56
Mineral supplement[$]		10	10	10
Vitamin supplement#		10	10	10
Binder		20	20	20
L-Methionine		-	1	1
Proximate composition*				
Moisture	20.93±0.02	4.24±0.03	5.49±0.03	5.31±0.18
Crude Protein, CP	30.84±0.38	40.27±0.45	39.98±0.37	40.16±0.39
Ether Extract, EE	33.10±0.24	18.63±0.27	18.56±0.14	17.68±0.20
Ash	10.30±0.18	14.30±0.28	14.20±0.23	14.13±0.31
Gross Energy (MJ kg^{-1})	n.d.[6]	22.10±0.11	22.30±0.03	21.28±0.06
Fatty acid composition*				
SFA	65.30±2.05	33.76±0.14	42.13±4.51	48.24±1.67
MUFA	28.37±0.89	32.87±1.23	34.46±0.61	33.04±1.45
PUFA	6.34±0.41	33.00±1.41	23.00±1.14	18.50±2.12
PUFA n3	0.66±0.04	22.01±0.45	13.71±0.22	10.11±0.33
PUFA n6	5.68±0.21	11.33±2.20	9.68±1.56	8.62±1.50
n9	18.07±1.14	26.50±0.71	28.00±1.41	25.50±2.12
EPA	0.20±0.02	6.85±0.24	4.24±0.49	3.12±0.74
DHA	-	13.42±0.89	8.24±0.36	5.97±0.72
n3/n6	0.12±0.10	1.99±0.43	1.44±0.25	1.19±0.17

[1]Bioceval GmbH & Co. KG Cuxhaven, Germany.
[2]Lombarda trading srl, Casalbuttano & Uniti (CR, Italy).
[3]SmartBugs srl (Treviso, Italy).
[4]Sacchetto spa (Torino, Italy).
[5]Consorzio Agrario (Pordenone, Italy).
[$]Mineral supplement composition (% mix): $CaHPO_4*2H_2O$, 78.9; MgO, 2.725; KCl, 0.005; NaCl, 17.65; $FeCO_3$, 0.335; $ZnSO_4*H_2O$, 0.197; $MnSO_4*H_2O$, 0.094; $CuSO_4*5H_2O$, 0.027; Na_2SeO_3, 0.067.

#Vitamin supplement composition (% mix): thiamine HCL Vit B1, 0.16; riboflavin Vit B2, 0.39; pyridoxine HCL Vit B6, 0.21; cyanocobalamine B12, 0.21; niacin Vit PP, 2.12; calcium pantotenate, 0.63; folic acid, 0.10; biotin Vit H, 1.05; myoinositol, 3.15; stay C Roche, 4.51; tocoferol Vit E, 3.15; menadione Vit K3, 0.24; Vit A (2500 UI kg^{-1} diet), 0.026; Vit D3 (2400 UI kg^{-1} diet), 0.05; choline chloride, 83.99.
*Values reported as mean of triplicate analyses.
[6]n.d.: not determined.
SFA = C10:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C24:0. MUFA= C16:1n9 + C16:1n7 + C18:1n9 + C18:1n7 + C20:1n9 + C22:1n9 + C24:1n9. PUFA= C18:2n6 + C18:3n3 + C18:3n6 + C20:2n6 + C20:3n3 + C20:3n6 + C20:4n6 + C20:5n3 + C22:6n3. PUFA n3 = C18:3n3 + C20:3n3 + C20:5n3 + C22:6n3. PUFA n6 = C18:2n6 + C18:3n6 + C20:2n6 + C20:3n6 + C20:4n6.

2.2 Chemical composition of feeds

The proximate composition and energy level of the H prepupae meal and of the experimental diets are shown in Table 1. Feed samples were analysed for moisture (AOAC #950.46), crude protein, CP (AOAC #976.05), ash (AOAC #920.153) and ether extract, EE (AOAC #991.36) contents according to AOAC International [44]. The gross energy content (GE) was determined using an adiabatic calorimetric bomb (IKA C7000, Werke GmbH & Co., Staufen, Germany).

The total lipid fraction of the H prepupae meal and of the three test diets was extracted using chloroform-methanol (2:1 v:v) mixture [45]. The fatty acid methyl esters (FAMEs) were obtained following the protocol described in Morrison and Smith [46] and quantified by gas chromatography (Varian 430-GC, FID) according to Tulli et al. [47] using tricosanoic acid (C23:0; Supelco, Bellefonte, PA, USA) as an internal standard.

The grading inclusion level of H meal resulted in an increase of total saturated fatty acids (SFA) and a decrease in total polyunsaturated fatty acids (PUFA) percentage in the experimental diets as shown in table 1.

2.3 Fish rearing conditions

The fish feeding trial was conducted at the Experimental Facility of the Di4A (Pagnacco, Udine, Italy; code 068UD047). The experimental protocol was designed according to the guidelines of the current European and Italian laws on the care and use of experimental animals (Directive 2010/63/EU, recognized in Italian legislation (D.L. 116/92)) and approved by the University of Udine Ethical Committee (Prot. N. 1/2018). All the handling procedures and sampling methods used in this trial followed the guidelines of the European Union directive 2010/63/EU on the protection of animals used for scientific purposes.

Two hundred and seventy juvenile rainbow trout (Oncorhynchus mykiss) with an initial body weight of 137.3 ± 10.5 g were randomly allocated to nine 1 m^3 square fiberglass tanks (30 specimens/tank). Tanks were connected to an open flow-through artesian well water system ensuring an approximate constant temperature of 13 °C, known to be near the thermal optimum for rainbow trout

rearing. After stocking, fish were fed a commercial diet and were given two weeks to adapt to the experimental conditions. At the end of this period, the 9 tanks were assigned to the three experimental diets (H0, H25 and H50) according to a random design with triplicate groups (tanks) per treatment. Fish were hand-fed the experimental diets over 98 days, in one daily meal (9:00 am) at 1.3% ratio of the total biomass, according to Stadtlander et al. [48]. Every three weeks, fish were group/tank weighed to adjust feeding rations. During the feeding trial, water quality parameters were monitored and recorded: temperature 12.8 ± 0.6 °C, dissolved oxygen 8.9 ± 0.43 mg L-1, pH 7.8 ± 0.2, total ammonia nitrogen 0.13 ± 0.02 mg L-1, nitrite-nitrogen <0.015 mg L-1, phosphorus 1.08 ± 0.57 mg L-1.

2.4 Tissue sampling and calculations

At the end of the feeding trial, after a 10 h fasting period, all fish were subjected to stage 3 anaesthesia with MSS-222 (300 mg L-1). Biometry measurements (standard length, cm and body weight, g) and growth performance indexes such as Fulton's condition factor (K), specific growth rate (SGR), weight gain (WG) and feed conversion ratio (FCR) were calculated per each fish/tank as follows:

$$K = [\text{fish weight (g)} / \text{fish standard length}^3] \times 100$$

$$SGR\ \% = 100 \times [(\ln \text{final body weight} - \ln \text{initial body weight}) / \text{number of feeding days}]$$

$$WG\ \% = 100 \times [(\text{final body weight} - \text{initial body weight}) / \text{initial body weight}]$$

FCR= total feed consumed (g) per tank biomass / weight gained (g) per tank biomass

Three fish per tank (9 fish per dietary treatment) were sacrificed by an overdose of the same anaesthetic and blood samples (approximatively 2 mL) were immediately withdrawn from caudal veins by heparinised syringes, stored on ice and centrifuged at 1,500 × g for 15 min at 4 °C. The obtained plasma was stored at -80 °C for subsequent metabolic parameter determinations. After blood sampling, liver (Li.) and digestive tract were immediately excised and washed with a 0.9% saline solution to remove the content. The digestive tract was quickly divided into medium intestine (M.I., corresponding to the tract immediately behind the anterior segment to the ileorectal valve) and hind intestine (H.I., from the ileorectal valve to the terminal part, excluding the rectum). Collected tissue (Li., M.I. and H.I.) were immediately put in individual plastic tubes, frozen in liquid N and stored at -80 °C for growth, stress and inflammatory gene expression analyses. In addition, subsamples of the same tissues were also quickly fixed in Bouin solution (Merk Sigma Aldrich, Milan, Italy) for histological analysis or frozen on dry ice (only Li.) for subsequent FTIRI analysis.

2.5 Plasma metabolic parameters

The plasma cholesterol (Chol, mg dL−1), triglycerides (Trig, mg dL−1), glucose (Glu, mg dL−1), total proteins (TP, g dL−1) and albumin (Alb, g dL−1) contents were determined using an automated analyser system for blood biochemistry (Roche Cobas Mira, Biosys, Milan, Italy) and commercially available kits (Biochemical Enterprise, Milan, Italy), following the manufacturer's protocols.

2.6 RNA extraction and cDNA synthesis

Total RNA was extracted from liver (L) and intestine samples (M.I. and H.I., approximately 90 mg) using RNAzol®RT reagent (Sigma-Aldrich®, R4533, Milan, Italy) and following the manufacturer's instructions. RNA concentration and integrity were analysed using NanoPhotometer® P-Class (Implen, Munich, Germany) and GelRed™ staining of 28S and 18S ribosomal RNA bands on 1% agarose gel, respectively. After extraction, complementary DNA (cDNA) was synthesised from 3 µg of total RNA the High Capacity cDNA Reverse Transcription Kit (Bio-Rad, Milan, Italy), following the manufacturer's instruction, diluted 1:10 in RNase-DNase free water and stored at −20 °C until quantitative real-time PCR (qPCR). An aliquot of cDNA was used to check primer pair specificity.

2.7 Real time PCR

PCRs were performed in an iQ5 iCycler thermal cycler (Bio-Rad, USA) and each sample was analysed via RT-qPCR in triplicate. Reactions were set on a 96-well plate by mixing, for each sample, 1 µL cDNA diluted 1:20, 5 µL of 2×concentrated iQ™ Sybr Green (Bio-Rad, USA) as the fluorescent intercalating agent, 0.3 µM forward primer, and 0.3 µM reverse primer. The thermal profile for all reactions was 3 min at 95 °C, followed by 45 cycles of 20 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C. Fluorescent signal were detected at the end of each cycle and the melting curve analysis was performed to confirm that only one PCR product was present in these reactions. Relative quantification of the expression of genes involved in fish growth (insulin like growth factors, igf1 and myostatin, mstn1a) and stress response (glucocorticoid receptor, gr and 70-heat-shock protein, hsp70) in liver and inflammatory/immune response (interleukin 10, il-10, tumor necrosis factor, tnf-α and toll-like receptor 5, tlr-5) in intestine was performed using β-actin and 60S ribosomal RNA (60S) as housekeeping genes to standardize the results. The primers sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) and are summarised in Table 2. Amplification products were sequenced, and homology was verified. Negative controls revealed no amplification product and no primer-dimer formation was found in control templates. Data were analysed using the iQ5 optical system software version 2.0, including Genex Macro iQ5 Conversion and Genex Macro iQ5 files (all from Bio-Rad). Modification of gene expression was reported with respect to controls. Primers were used at a final concentration of 10 pmol µL-1.

Table 2. Oligonucleotide primers of each gene investigated in this study.

Gene name		Primer sequence		Annealing temperature (°C)	Gene bank accession number
		Forward	**Reverse**		
Liver	*igf1*	TGGACACGCTGCAGTATGTGTGT	CACTCGTCCACAATACCACGGT	60	GQ924783
	mstn1a	CCGCCTTCACATATGCCAA	CAGAACCTGCGTCAGATGCA	60	AY839106
	gr	GCCTTTTGGCATGTACTCAAACC	GGACGACTCTCCATACCTGTTC	60	AY549305
	hsp70	CCCTGGGCATCGAAACC	CCCTCGTAGACCTGGATCATG	60	AY423555
Intestine	*il-10*	CGACTTTAAATCTCCCATCGA	GCATTGGACGATCTCTTTCTT	59	DQ821115
	tnf-a	AGCATGGAAGACCGTCAACGAT	AGCATGGAAGACCGTCAACGAT	60	DQ070246
	tlr-5	GGCATCAGCCTGTTGAATTT	ATGAAGAGCGAGAGCCTCAG	57	NP001118216
	β-actin	AGACCACCTTCAACTCCATCAT	AGAGGTGATCTCCTTCTGCATC	60	AJ537421
	60S	TTCCTGTCACGACATACAAAGG	GTAAGCAGAAATTGCACCATCA	60	DT044641.1

2.8 Histology

Liver (Li.) and intestine (M.I., H.I.) samples were fixed by immersion in Bouin solution and stored at 4 °C for 24 h. Subsequently, samples were washed 3 times with 70% ethanol for 10 min and preserved in a new 70% ethanol solution. After dehydration by graded ethanol series, samples were washed with the clearing agent "Histo-Clear" (Bio-Clear, Bio-Optica, Milan, Italy) and embedded in paraffin (Bio-Optica, Milan, Italy). Paraffin blocks were cut with a microtome (Leica RM2125 RTS, GmbH, Wetzlar, Germany) and 5 µm sections were stained with Mayer haematoxylin and eosin Y (Sigma-Aldrich, Milan, Italy) and PAS (Periodic Acid of Schiff), following the manufacturer's instructions (Bio-Optica, Milan, Italy). Stained sections were examined under a Zeiss Axio Imager.A2 (Zeiss, Oberkochen, Germany) microscope and the images were acquired by means of a combined colour digital camera Axiocam 503 (Zeiss, Oberkochen, Germany). For the quantitative image analysis of intestinal folds morphometric evaluations, ten transversal sections of M.I. at 200 µm intervals, for each fish sample were analysed. This interval was chosen in order to avoid repetitions in the measurements of intestinal folds. All the undamaged and non-oblique folds (at least 150 measurements per fish) were measured using ZEN 2.3 software (Carl Zeiss Microscopy GmbH), and the measurements were reported as means ± standard deviation (SD).

2.9 Fourier Transform Infrared Imaging spectroscopy (FTIRI) measurements and data analysis

FTIRI spectroscopy has been recently optimized in order to study different topics in fish species [32,49] and the protocol described by Giorgini et al. [50] was adopted to analyse liver samples of Ho, H25 and H50 trout groups. A cryotome was used to cut, from each liver sample, three thin sections (≈10 µm

thickness) at 200 μm away from each other. No fixative was used. Sections were immediately deposited onto CaF_2 optical windows (1 mm thickness, 13 mm diameter) and air-dried for 30 min. All sections were analysed at the IR beamline SISSI, ELETTRA - Synchrotron (Trieste, Italy), within 48 h after cutting. A Bruker VERTEX 70S interferometer coupled with a Hyperion 3000 Vis-IR microscope and equipped with a liquid nitrogen-cooled two-dimensional FPA detector (detector area size 164×164 μm2, 64×64 pixels, pixel resolution 2.56 μm; Bruker Optics GmbH, Germany) was used. A 15x condenser/objective was employed to obtain the visible image on each section to identify the areas of interest. IR maps (164×164 μm2) were acquired in transmission mode in the spectral range 4000-700 cm^{-1} (4096 spectra; 256 scans; spectral resolution 4 cm^{-1}). Background spectra were acquired on clean portions of CaF_2 windows. Raw IR maps were submitted to the following pre-processing treatments: (i) Atmospheric Compensation routine (OPUS 7.1 software package) to correct the atmospheric contributions of carbon dioxide and water vapour, and (ii) vector normalization in the 3800-950 cm^{-1} spectral range, to avoid artefacts induced by local thickness variations (OPUS 7.1 software package). Pre-processed IR maps were then integrated under the following spectral ranges, to rebuild the topographical distribution of lipids, proteins and glycogen: 3000-2825 cm^{-1} (overall lipids, Lipids); 1720-1480 cm^{-1} (overall proteins, Proteins); 1180-1000 cm^{-1} (overall glycogen, Glycogen). An arbitrary colour scale was used with warm (red) to white colour indicating the highest absorbance value, while blue colour represented the lowest.

On all the spectra of each IR map, several bands with biological relevance were selected and the corresponding integrated areas calculated by using Integration routine (OPUS 7.1): 3000-2825 cm^{-1} (asymmetric and symmetric stretching modes of CH_2 and CH_3 groups mainly in lipid alkyl chains, LIP); 2943-2895 cm^{-1} (asymmetric stretching mode of CH_2 groups in lipid alkyl chains, CH_2); 1711-1483 cm^{-1} (Amide I and II bands of proteins, PRT); 1181-1142 cm^{-1} (stretching of C-O-H groups in carbohydrates, COH); 1073-1000 cm^{-1} (stretching of C-O and C-C moieties and bending of C-O-H groups in carbohydrates, mainly attributed to glycogen, GLY). The integrated areas of the spectral regions at 3000-2825 cm^{-1} and 1767-950 cm^{-1} was added and taken as representative of the overall tissue biomass (TBM). The following band area ratios were then calculated: LIP/TBM (total amount of lipids); CH_2/TBM (total amount of saturated alkyl chains); CH_2/LIP (saturated alkyl chains with respect to total lipids); PRT/TBM (total amount of proteins); GLY/TBM (total amount of glycogen), and COH/TBM (total amount of carbohydrates).

The average IR absorbance spectra of H0, H25 and H50 liver trout groups were also calculated in the spectral range from 4000 to 900 cm^{-1} and converted to second derivative mode, to obtain the position of the most meaningful absorption bands.

2.10 Statistical analyses

Data are presented as mean value ± SD. Data were tested for normality and homogeneity of variances by using Shapiro-Wilk's and Levene's tests, respectively. One-way ANOVA was adopted for growth parameters where the

tank (fish group) was the experimental unit. In case of plasma metabolites, where the individual fish was the experimental unit, data analysis was carried out using a mixed ANOVA model, including the tank as a random variable. When appropriate, the Tukey's post hoc test (significant level $p < 0.05$) to detect significant differences among the dietary treatments was used. All analyses were performed by using the SPSS package (SPSS Inc., Chicago, IL, USA). For the gene expression and FTIRI results, the statistical software package Prism5 (GraphPad software) was used and one-way analysis of variance was performed followed by Student's test for the comparison of M.I. and H.I. results.

3. Results

3.1. Fish growth

Fish growth performance parameters are reported in Table 3. The survival rate of fish was 100% over 98 days of feeding trial for all the experimental groups. The fish readily accepted the experimental diets and all feeds were consumed without rejection or loss. Fish fed both diets containing insects (H25 and H50) resulted in a final body weight (FBW), K, WG, SGR and FCR that were not significantly different ($p > 0.05$) from those attained by fish fed the Ho diet.

Table 3. Growth response of rainbow trout fed the experimental diets.

	Ho	H25	H50
FBW (g)[1]	301.21±32.21	279.59±37.26	251.27±22.14
K[2]	1.13±0.11	1.13±0.01	1.12±0.07
WG (%)[3]	119.81±16.83	102.91±24.24	83.18±14.32
SGR (%)[4]	0.80±0.08	0.71±0.12	0.61±0.08
FCR[5]	1.02±0.17	1.22±0.35	1.47±0.28

Data are reported as mean of triplicate tanks and presented as mean ± SD.
[1]Final body weight
[2]Fulton's condition factor
[3]Weight gain
[4]Specific Growth Rate
[5]Feed Conversion Ratio

3.2. Plasma metabolic parameters

The plasma metabolic parameters obtained from samples collected at the end of feeding trial are reported in Table 4. Plasma cholesterol (Chol), triglycerides (Trig), glucose (Glu), total proteins (TP) and albumin (Alb) levels did not show significant differences among the dietary groups ($p > 0.05$).

Table 4. Plasma metabolic parameters measured in rainbow trout fed the test diets at the end of the 98-days feeding period.

Plasma parameters	Ho	H25	H50
Chol (mg dL^{-1})	188.0±20.2	178.6±29.9	196.7±40.8
Trig (mg dL^{-1})	212.4±50.1	186.8±55.6	202.5±37.8
Glu (mg dL^{-1})	112.7±16.8	103.6±9.9	123.0±31.1

TP (g dL^{-1})	3.5±0.5	3.2±0.6	3.4±0.7
Alb (g dL^{-1})	1.3±0.2	1.5±0.4	1.6±0.3

Chol, cholesterol; Trig, triglycerides; Glu, glucose; TP, total proteins and Alb, albumin. Values are reported as mean ± SD

3.3. Gene expression

In liver samples, at the end of feeding trial, real-time PCR analyses were performed on genes involved in fish growth (igf1 and mstn1a) and stress response (gr, hsp70).

Growth biomarkers, igf1 and mstn1a (Fig. 1a and 1b), did not show significant differences ($p > 0.05$) among the dietary treatments. The expression of the genes involved in stress response (gr and hsp70), gr did not show any significant difference among the experimental groups (Fig. 1c), while hsp70 showed a significant upregulation only in H50 dietary treatment ($p < 0.05$) (Fig. 1d).

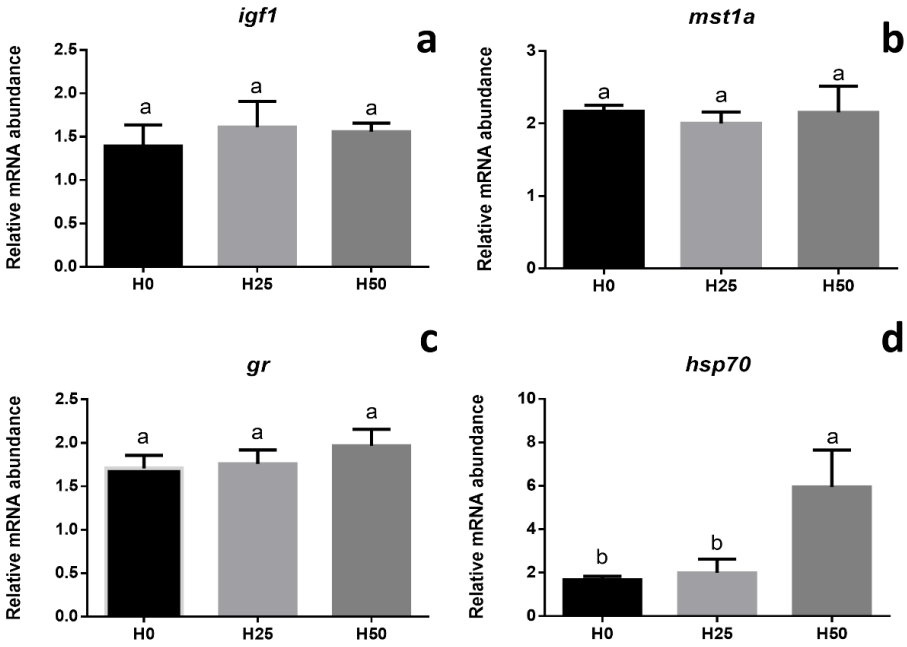

Fig. 1. Relative mRNA abundance of genes involved in fish growth (igf1 and mstn1a) and stress (gr and hsp70) analysed in liver from trout fed diets including different H meal levels (H0, H25, H50). Different letters indicate significant differences among the experimental groups ($p < 0.05$). Values are presented as mean ± SD.

The inflammatory response was investigated in the medium (M.I.) and hind (H.I.) intestine through the gene expression of cytokines (il-1o, tnf-α), while the innate immune defense was analysed by monitoring the membrane receptor tlr-

5. The most significant differences were shown in the M.I. (Fig. 2). In particular, a significant ($p < 0.05$) higher gene expression of il-10 (Fig. 2a), tnf-α (Fig. 2b) and tlr-5 (Fig. 2c) was shown in both H25 and H50 groups compared to H0 group. As regards the expression of the same genes in the H.I., no significant differences were observed among the three experimental groups, except for a higher il-10 gene expression in H25 group (Fig. 2d).

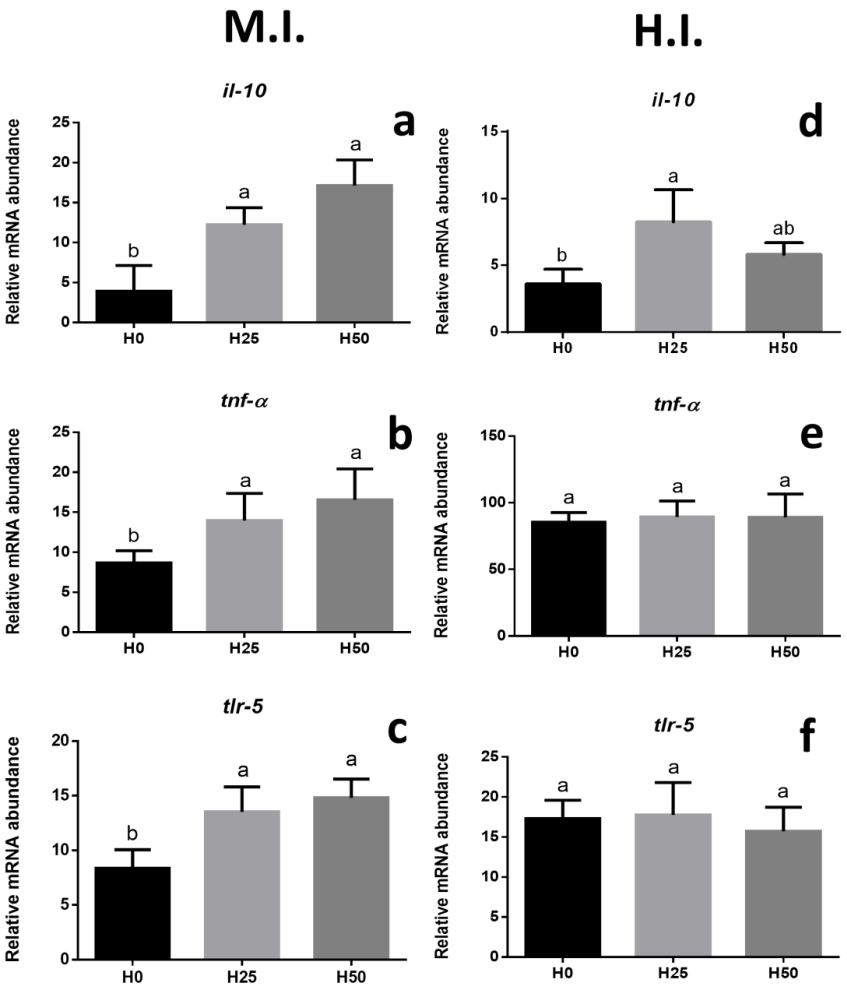

Fig. 2. Relative mRNA abundance of genes involved in inflammatory response and innate immune defense, analysed in the medium (M.I.) and hind (H.I.) intestine. Different letters indicate statistically significant differences among experimental groups ($p < 0.05$). Values are presented as mean ± SD.

3.4. Histology

Histological analysis of the liver stained with periodic acid of Shiff (PAS) is reported in Fig. 3. H50 (Fig. 3e, f) showed an increase in liver lipid accumulation compared to both Ho (Fig. 3 a, b) and H25 (Fig. 3c, d). No appreciable differences in glycogen accumulation were observed between the dietary treatments by means of PAS staining.

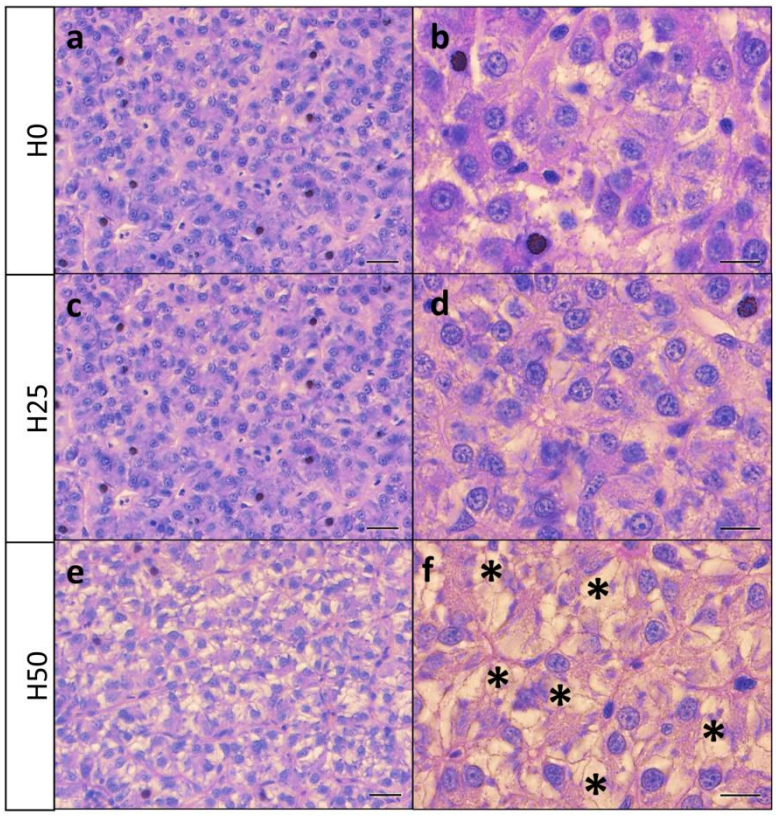

Fig. 3. Liver histology of rainbow trout stained with periodic acid of Shiff (PAS). Asterisks indicate lipid accumulation. Scale bars: a, c, e = 20 μm; b, d, f = 10 μm.

Concerning the histological analysis of the different intestinal tracts (M.I. and H.I.), conventional haematoxylin and eosine staining (HE) was performed to investigate possible inflammatory alterations while periodic acid of Shiff staining (PAS) was used to highlight differences in mucous cells distribution (Fig. 4). No inflammatory events were shown in both M.I. and H.I. of all fish examined (Fig. 4a, c, e, g, i, m). On the contrary, an appreciable increase in mucous cells

(hyperplasia) was shown in the H.I. tract of fish fed H50 (Fig. 4 n) compared to both Ho and H25.

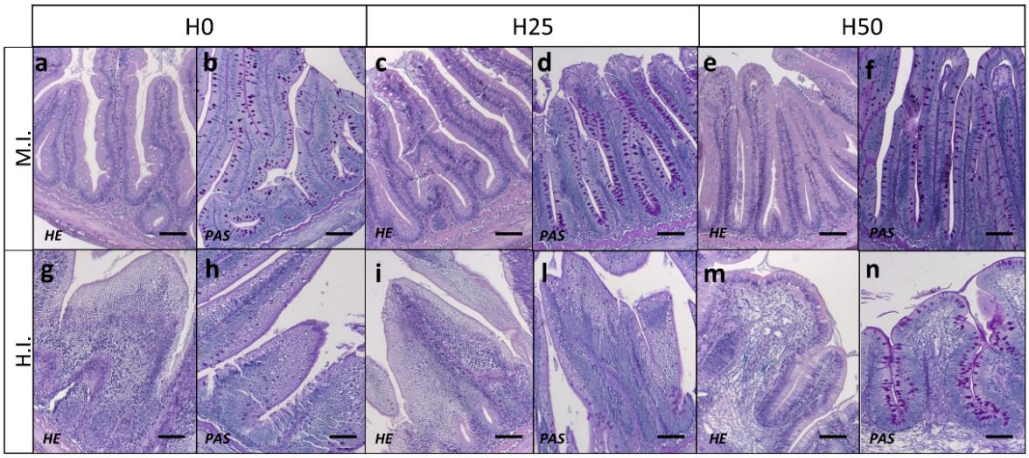

Fig. 4. Histology of medium (M.I.) and hind (H.I.) intestine from the different feeding groups. Section were stained with hematoxylin and eosine (HE) and periodic acid of Shiff (PAS). Arrow heads indicate mucous cells hypertrophy. Scale bar = 100 μm.

Finally, the morphometric evaluation of the intestinal fold length was performed in the medium tract of the intestine (M.I.) to evaluate a possible reduction of the absorptive epithelial surface. Measurements evidenced a significant shortening of the fold length in fish fed diets containing insects (697.5 ± 15.9 μm and 681.1 ± 11.2 μ for H25 and H50, respectively) compared to fish fed control diet Ho (821.7 ± 36.8 μm) ($p < 0.05$).

3.4. FTIRI analysis

In Fig. 5, the average spectra of liver sections of all experimental groups were reported in absorbance and second derivative modes. In Table 5, the most relevant absorption bands are listed together with the vibrational meaning and the biochemical assignment, which were done according to the literature [35,48]. Modifications in the spectral profiles were detected mainly in the regions associated with the vibrational modes of lipid alkyl chains (3000-2824 cm^{-1}) and carbohydrates (1180-1000 cm^{-1}).

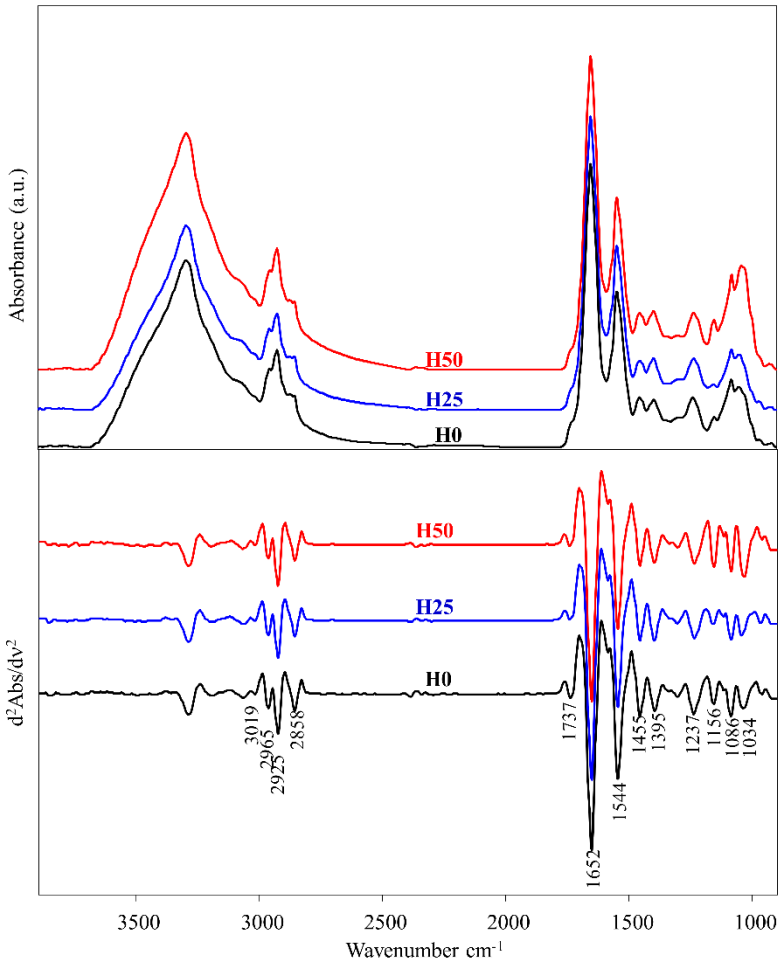

Fig. 5. Average spectra of liver sections of rainbow trout fed the test diets: Ho (black), H25 (blue) and H50 (red). Spectra were reported in the 4000-900 cm−1 spectral range in absorbance and second derivative modes (the wavenumbers of the most relevant peaks are reported in the bottom part).

A preliminary imaging analysis was performed on IR maps to retrieve information on the topographical distribution and the relative amount of Lipids (Fig. 6b), Proteins (Fig. 6c) and Glycogen (Fig. 6d) in liver sections from the different experimental groups. H50 liver samples, exhibited higher amounts of Lipid and Glycogen, together with a moderate decrease of Proteins than liver of fish fed Ho; in H25, only an increase in total Lipids was detected compared to Ho (Fig. 6b), while Proteins and Glycogen showed similar amounts.

Table 5. Absorption bands detected on second derivative IR spectra of H0, H25 and H50 experimental groups. For each band, the wavenumber (expressed as cm-1), together with the vibrational mode and the biological meaning are reported.

Wavenumber	Vibrational mode	Biological meaning
~3019	Stretching vibration of =CH groups	Mainly lipid alkyl chains
~2965,~2925,~2858	Asymmetric and symmetric stretching vibrations of CH_3 and CH_2 groups	
~1737	Stretching vibration of C=O ester moieties	
~1662,~1544	Amide I and II bands	Proteins
~1455, ~1395	Bending vibrations of proteins side chains	
~1237, ~1087	Asymmetric and symmetric stretching vibrations of PO_2- groups	Phosphate groups
~1156	Stretching vibrations of C-O-H moieties	Carbohydrates and glycogen
~1034	Stretching vibrations of C-O and C-C moieties and bending of C-O-H groups	

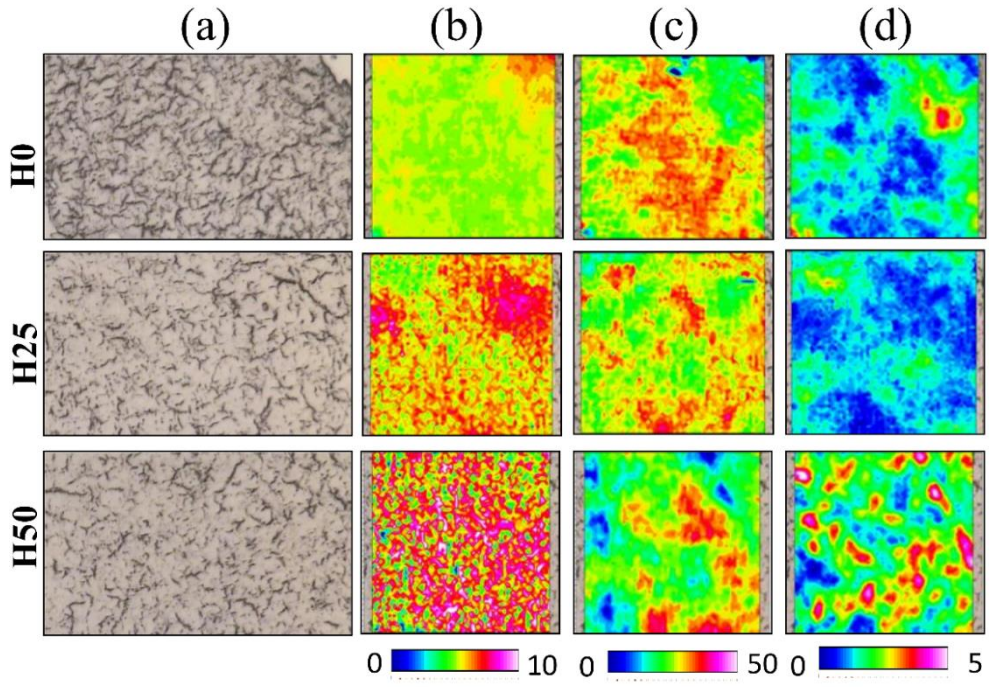

Fig. 6. Microphotographs (a) of representative liver sections of H0, H25 and H50 groups analysed by FTIRI and topographical distribution of Lipids (b), Proteins (c) and Glycogen (d). Colours from warm (red) to white indicate higher absorbance values, whilst blue colour indicates the lower ones. See colour scale on the bottom.

The semi-quantitative analysis performed on specific band area ratios allows for making the following considerations (Fig. 7): (i) the relative amount of lipids calculated on tissue biomass (LIP/TBM) resulted in significantly increased H25 and

H50 compared to Ho ($p < 0.05$) (Fig. 7a); (ii) the relative amounts of saturated lipid alkyl chains calculated both on tissue biomass (CH_2/TBM) and on total lipids (CH_2/LIP) were significantly lower in H25 and H50 compared to Ho ($p < 0.05$) (Fig. 7b and 7c); (iii) a significant decrease of the relative amount of proteins calculated on tissue biomass (PRT/TBM) was detected only in H50, with respect to Ho and H25 ($p < 0.05$) (Fig. 7d); (iv) the highest levels of glycogen and carbohydrates, calculated on tissue biomass (GLY/TBM and COH/TBM, respectively), were found in H50, and the lowest in H25 ($p < 0.05$) (Fig. 7e and 7f).

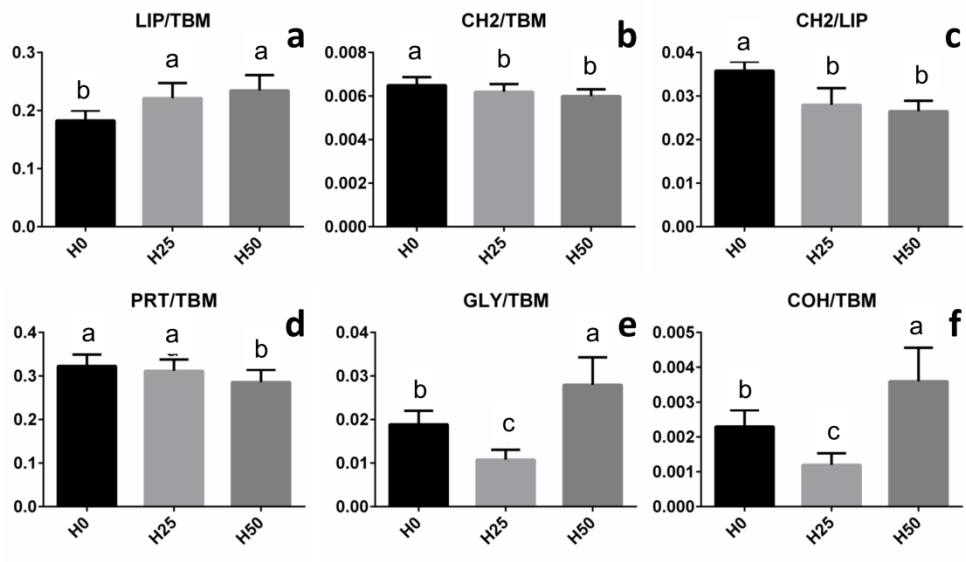

Fig. 7. Statistical analysis of band area ratios calculated on Ho, H25 and H50 liver samples: LIP/TBM, CH_2/TBM, CH_2/LIP, PRT/TBM, GLY/TBM and COH/TBM. Values are presented as mean ± SD. Different letters indicate statistically significant differences among the experimental groups ($p < 0.05$).

4. Discussion

In the present study, inclusion of full-fat *Hermetia illucens* meal in practical diets for rainbow trout resulted in a moderate but not significant reduction of growth and FCR. These findings are in agreement with several previous studies which tested inclusion levels (≤ 50%) of black soldier fly (BSF) in the same [21,48] and different fish species, such as channel catfish (*Ictalurus punctatus*) [51], Atlantic salmon (*Salmo salar*) [26,52], Jian carp (*Cyprinus carpio*) [36], turbot (*Psetta maxima*) [14], gilthead seabream (*Sparus aurata*) [53], European sea bass (*Dicentrarchus labrax*) [24], Nile tilapia (*Oreochromis niloticus*) [54] and yellow catfish (*Pelteobagrus fulvidraco*) [55].

Nevertheless, results on full-fat or defatted *Hermetia* meal inclusion in aquafeeds are still contradictory. For example, in rainbow trout, dietary inclusion level of 30% or 33% of full-fat *Hermetia* prepupae meal caused a worsening in WG and FCR values [19,20]. Similarly, an inverse relationship was observed in trout fed increasing dietary amount of defatted BSF meal both on thermal-unit growth coefficient and FCR [56]; this was also evidenced in juvenile turbot [14]. These contradictory results may be related to several factors such as the BSF dietary inclusion level, the use of full-fat or defatted insect meal, the presence of chitin, the feeding regime to which fish were subjected (restricted *vs* apparent satiation), the manufacturing of the feeds (pelleting *vs* extrusion) and, of course, the fish species investigated as well as the stage of development (juvenile *vs* adult). Therefore, further studies are needed to better elucidate the physiological responses of fish to these new diets, including the species-specificity of these responses.

Since growth and ontogeny follow a genetically programmed and well-defined sequence in which gene transcription and hormone regulation are crucial, clinical and zootechnical parameters may not be sufficient to monitor fish growth and development. Therefore, besides the traditional markers (morphological, histological, physiological and biochemical), it may be important to look for alternative ones at the molecular level. Consequently, gene expression can be used to generate useful insights linking biotic and abiotic conditions to individual performances. Molecular markers can be identified among those genes whose expression could reasonably be modified by different conditions, including nutrition [57,58] and, among these, several studies showed that *igfs* and *mstn* are useful growth biomarkers. Nutritional deficiencies have deep impact on fish growth and welfare and circulating IGFs levels are known to be nutritionally regulated [33,35,59,60]. In fact, in many fish species, IGFs blood or tissue levels mRNA positively correlate with feeding levels, dietary protein content and body growth rate [95]. On the other hand, myostatin, a member of the transforming growth factor-β (TGF-β) family, is considered to be an inhibitory factor in the growth of skeletal muscle [61]. Therefore, fish final growth is related to the interplay of these positive and negative signals [62]. In the present study, biometric results were fully supported by the molecular ones since no significant differences in *igf1* and *mstn1a* gene expression were detected among the experimental groups.

Captive rearing, including nutrition, is quite different from a natural condition, often causing a decline in fish welfare. The Fulton's condition factor (K), commonly used to describe fish well-being, is based on the assumption that for a given length, heavier fish are in better condition [63]. K values less than 1 indicate unhealthy fish, while values higher than 1 denote fish in a good physiological state. Independently from the dietary treatment, the K values reported in the present trial were higher than 1 and similar to those recorded in a previous study carried out in adult rainbow trout fed defatted *Hermetia* meal [21]. Farmed fish are usually exposed to a variety of stressors and long-term exposure has negative effects on fish health and performance, by increasing

disease susceptibility and decreasing fish growth [64]. However, to our knowledge, scant information is available for rainbow trout regarding the effects of insect meal feeding on the expression of stress and immune-related genes, as well as on the levels of serum metabolites as secondary and tertiary stress responses, respectively [65].

The results of the present study have demonstrated that a 98-days feeding trial with graded dietary inclusion level of full-fat insect meal had no significant effects on the activation of the primary stress response (*gr*) gene expression in trout. At a cellular level, the stress response is often mediated by heat-shock proteins (HSPs), a family of highly conserved proteins present in all cells and life forms [66]. A variation in HSPs gene expression is often considered a useful bio-indicator in stress response [67].

An up-regulation of the *hsp70* gene expression was observed, at hepatocyte level, in fish fed the highest insect meal dietary inclusion (H50) possibly suggesting a physiological activation of stress/inflammation response after 98 days of feeding trial. Similarly, a significant up-regulation of *hsp70* was observed in Jian carp fed a diet including defatted BSF larvae higher than 79 g kg^{-1}, suggesting a potential stress response induction [36]. On the contrary, in Atlantic salmon [68] *hsp70* gene expression was not affected by the inclusion of 600 g kg^{-1} of defatted insect meal. The present contradictory results obtained in these trailblazing studies deserve further investigation in order to clarify the stress response of fish fed diets including insect meal. Anyway, considering the plasma metabolic profile of trout fed graded inclusion level of full-fat *Hermetia* meal, no significant differences were noted in the parameters herein considered, which were in line with those previously found in the same species [69]. The inconsistency of our results and those observed in other fish species, such as European seabass [24], deserves to be further investigated in order to better clarify the potential ipo-cholesterolemic effect of insect meal.

As a primary site of food digestion and nutrient absorption, the gastrointestinal system plays a key role in the *optimum* utilization of dietary nutrients, which depends on its functionality. Previous studies on alternative feed ingredients show that high inclusion levels of plant protein as FM replacer can affect both gut and liver integrity [70]. Liver plays a key role in many fish metabolic pathways and its morphological structure, macromolecular composition and gene expression are deeply influenced by the diet [33-35]. In this regard, the present study, demonstrates that stress biomarkers are affected by diets including insects. Even if the offered diets were isolipidic, the lipid hepatic accumulation detected by histological and FTIRI analyses was very different. Specifically, fish fed diets including insect meal showed an increase in lipid accumulation compared to control fish (*Lipids* and LIP/TBM). FTIRI analysis provided deeper insight into liver biochemical composition, revealing that most of the accumulated lipids showed shorter alkyl chains (CH_2/TBM and CH_2/LIP). In addition, a high accumulation of glycogen (*Glycogen* and GLY/TBM) and, in general, of carbohydrates (COH/TBM), was detected in trout liver fed the diet with the highest insect meal inclusion.

Finally, no relevant changes in the protein pattern (*Proteins* and PRT/TBM) were observed; when the highest insect meal inclusion level was used, only a moderate decrease of proteins was found.

A recent paper by Zarantoniello et al. [59] reported that an increase in the dietary SFA may play an important role in the development of hepatic steatosis [71], causing liver dysfunction by promoting endoplasmic reticulum stress and apoptosis [72,73] and these results are in line with the *hsp70* up-regulation observed in fish fed the H50 diet. Aside from the deficiencies in fatty acid composition, insect meal is characterized by the presence of chitin, a polysaccharide that constitutes the insect and crustacean exoskeleton. The specific role of chitin in fish diets is still controversial and is related to its dietary level of inclusion; when included at low levels, it might act as an immune-stimulant and anti-inflammatory molecule in fish [74], while if included at high doses it might reduce fish growth and intestinal inflammation [14,36]. In addition, the role of chitin seems to be size dependent: large chitin polymers may be inert; fragments of 40–70 µm may be pro-inflammatory and the smaller ones (less than < 40 µm) anti-inflammatory [75]. Consequently, when using diets including chitin, the gastrointestinal tract should be carefully analysed, possibly through a multidisciplinary approach.

Intestinal morphology is a valuable means to assess both gut health and its functional *status* [32]. In the present study, the histological analyses performed in the medium and hind intestine highlighted the absence of severe inflammatory events but evidenced the presence of goblet cells that produce neutral mucins, as previously observed in trout fed defatted insect meal [27,76] and in black tetra (*Gymnocorymbus ternetzi*) [77]. Mucus-secreting cells are normally observed in the mucosa of digestive tract of fish, with distribution and histochemical characterization depending on species, age, gut segment, diets and feeding habits [27]. In the present study, the mucin type cells were studied along the intestinal tract [76] and an increasing amount of mucin goblet cells were specifically observed in the hind gut tract of fish fed the highest *Hermetia* meal inclusion (H50). These results are in agreement with previous observations in trout [27], tiger barb (*Puntius tetrazona*), black tetra [77] and in rice field eel (*Monopoterus albus*) [78], in which the accumulation of mucin cells in the posterior intestine was associated with intestinal protection and lubrification [77]. Dietary manipulation is also known to influence the intestinal microvilli structure [79] and, in order to assess possible gut histopathological evidences, villi length was investigated [36,58,79]. Since proteins are mainly digested and absorbed in the proximal and mid intestine, and to a lesser extent in the hind tract (H.I.), the medium portion of the intestine (M.I.) was analysed. The present study evidenced a negative effect on M.I. *villus* length in fish fed insect including diets showing both a reduction in the absorptive epithelial surface and a potential occurrence of gut inflammation [36,37]. These histological observations were fully supported by the gene expression analyses (which can be useful to precociously detect physiological responses) of the immune related genes [80], showing a significantly higher gene expression in M.I. respect to the other intestinal tracts

analysed. Similar negative effects were previously observed in Jian carp [36] fed a diet including defatted BSF larvae meal up to 79 g kg^{-1} resulted in irregularity of gut microvilli shape. However, other studies performed on Salmonids evidenced no significant histological modifications at intestinal level [21,26,27].

In this study, graded levels of full-fat *H. illucens* dietary meal inclusion were associated with increasing levels of purified plant protein-rich ingredients, such as pea protein concentrate and wheat gluten, in order to keep all diets isonitrogenous and isoenergetic. Even if interactive effects between protein sources of different origin cannot be ruled out [81], they were expected to be minor in magnitude, hence supporting the idea that changes in fish responses were basically due to the graded levels of insect meal in the diet. In fact, in this experiment, both pea protein concentrate and wheat gluten were included at levels that were shown not to alter growth and gut health of Salmonids when used either singly or in combination [39-42].

5. Conclusions

In conclusion, the present study highlighted that a dietary inclusion of full-fat *H. illucens* meal up to 210 g kg^{-1} in a practical diet for rainbow trout did not hamper fish growth; however, the application of a multidisciplinary approach evidenced that some biomarkers related to stress and immune function were altered, as well as the liver macromolecular composition.

Further investigations are needed to deepen the knowledge of dietary manipulation and mechanisms responsible for potential combined effects when insect meal and other plant protein sources are used in practical diets for rainbow trout.

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Paper 8

Dietary inclusion of full-fat *Hermetia illucens* prepupae meal in practical diets for rainbow trout (*Oncorhynchus mykiss*) do not impair fillet quality: Lipid metabolism investigations

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Abstract

Insects are able to bioconvert organic waste into protein and lipid sources usable in aquafeed formulation, in a circular economy fashion. *Hermetia illucens* (H) is particularly interesting for its nutritious traits, although, unfortunately, it is mainly composed of saturated fatty acids (SFA), while n-3 polyunsaturated fatty acids (PUFA) are scarce and this FA profile impinges on fish FA profile.

The present study undertook an interdisciplinary approach to explore the effects of diets for rainbow trout (*Oncorhynchus mykiss*) containing full-fat H meal on qualitative traits of fillets, liver FA composition, as well as on the expression of genes related to lipid metabolism in liver, pyloric caeca and mid intestine. Juvenile fish were fed either of three isoproteic, isolipidic and isoenergetic diets: one control diet based on fishmeal and purified protein-rich vegetable ingredients with 0% H inclusion (H0) and two experimental diets with graded substitution levels of H meal at 25% (H25) and 50% (H50) in place of fishmeal. Despite the FA profile of the three diets differed depending on the H meal presence, not only did biometrics and physical traits of fillets not worsen, but also total lipids and FA profile of fillets were not significantly affected by the different dietary regime, except for SFA. Total SFA, C12:0 and C14:0 increased with the increasing dietary substitution of H meal ($p<0.01$, $p<0.001$, $p<0.01$, respectively), while C16:0 showed an opposite trend ($p<0.01$). The sum of eicosapentaenoic (EPA) docosahexaenoic (DHA) acids was in average 14.92 g FAME/100 g total FAME. Hepatic total lipid content was not affected by the different diets, while FA profile seemed to deeply resemble the FA profile of the diets. However, DHA did not significantly differ between the groups and showed a mean value of 11.07 g FAME/100 g total FAME. Interestingly, *fads2* gene

expression of pyloric caeca increased in fish fed diets containing full-fat H meal (Ho *vs* H50: $p<0.05$); no significant differences in the expression of *elovl1* and *elovl2* were observed in the different gastrointestinal tracts considered (L, C and M), although trends were noticed.

In conclusion, full-fat H meal slightly affected rainbow trout qualitative traits, guaranteeing a nutritionally healthy food, while it had some effects on lipid metabolism, as suggested by liver FA profile and pyloric caeca, mid intestine and liver gene expression. Future studies on the biological mechanisms behind the macroscopic traits of fish fed insects are warmly encouraged.

Key words: rainbow trout, *Oncorhynchus mykiss*, black soldier fly, *Hermetia illucens*, lipid metabolism, gene expression, qPCR, quality.

1. Introduction

At date, insects are very studied ingredients for aquafeed formulation (Henry et al., 2015; Nogales-Mérida et al., 2018; Sánchez-Muros et al., 2014). The main reason of their use in feed formulation lies in the environmental, economic and societal benefits that insect production and use is supposed to generate. Insects are optimal bioconvertors since they can be reared on by-products such as organic waste producing protein and lipid sources that in turn can be used in aquafeed formulation, fostering the circular economy concept (van Huis et al., 2013). The black soldier fly, *Hermetia illucens* (H), is one the most studied insect species in aquafeed formulation especially for its biochemical characteristics that make it nutritious for fish (Barroso et al., 2014; Makkar et al., 2014; Sánchez-Muros et al., 2014).

Insect lipids are primarily composed of saturated (SFA) and monounsaturated fatty acids (MUFA), while n-3 polyunsaturated fatty acids (PUFA) are poorly present. This aspect is extremely important in fish nutrition since this deficiency may affect fish biochemical composition (Henry et al., 2015). In fact, fish are renowned for their high content of long-chain PUFAn-3 (LC PUFA), which are mainly accumulated through the diet and, minimally, synthesised by endogenous production (especially in fresh water species) (Tocher, 2003). LC PUFA like eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) are well known to reduce the risk of cardiovascular and inflammatory disorders and depression (Rosenlund et al., 2010) and thus the aquaculture production must guarantee the presence of these fatty acids in the final product. As a consequence, the aquaculture industry is continuously looking for "suitable, sustainable, and environmentally acceptable" dietary alternatives to fishmeal and fish oil, ensuring high levels of EPA and DHA in the final product (Tocher, 2003), in addition to guaranteeing its set of physical, chemical and sensorial attributes.

Recently, several feeding trials fed salmonids with different dietary inclusion levels of H meal, showing that both digestibility and growth performance are

usually not negatively affected (Lock et al., 2016; Renna et al., 2017). However, a potential effect of this new ingredient was found on fillet qualitative traits (Belghit et al., 2019, 2018a; Mancini et al., 2017; Renna et al., 2017). Even if commercial insect meal usually undergoes partial defatting by the feed industry, at the end of the process insect meal still contains 8-15% of total lipids, which are included in fish feed and assimilated by fish (Borgogno et al., 2017; Sealey et al., 2011).

The FA profile of the final product (fillet or skin) is the macroscopic manifestation of complex metabolic processes occurring between feed ingestion and lipid deposition in the fillet. Nutrient absorption in fish takes place along the entire intestinal tract, usually decreasing along the organ in rainbow trout and Atlantic salmon (Bakke et al., 2010; NRC, 2011). The enzyme encoded by *cd36* gene is involved in cellular FA uptake by enterocytes, hepatocytes, adipocytes, … (The UniProt Consortium, 2019). The chyme contains emulsified lipids and lipid-soluble vitamins, which downstream are further emulsified by bile acids and consequently hydrolysed, absorbed and then re-esterified into triacylglycerol. Pyloric caeca execute the absorption of several lipidic components, such as free FAs, lysophospholipids, monoacylglycerols (Turchini et al., 2009), as well as *de novo* LC PUFA production (Bell et al., 2003) by means of elongase (*elovl*) and desaturase (*fads*) enzymes. Scant investigations on mid intestine FA catabolism and *de novo* synthesis are found in the literature (Lazzarotto et al., 2018; Tacchi et al., 2012), however, pyloric caeca are thought to play an important role in DHA synthesis (Bell et al., 2003).

More grounded information is available when it comes to the functions of hepatocytes. Among the numerous functions, they receive nutrients coming from the intestine through the circulatory system, store lipids (Bakke et al., 2010; NRC, 2011) and are the main district dedicated to FA metabolism (Tocher, 2003). FA metabolism is governed by a substantial number of genes, in turn regulated by endogenous and exogenous conditions, for instance dietary lipids. $fads_s$, $elovl_s$ and peroxisome proliferator-activated receptors ($ppar_s$) are some of those genes and are expressed in at least liver, adipose tissue and muscle. $ppar_s$ are transcriptional regulators that are potentially expressed in all tissues and control the expression of genes playing roles in storage, mobilisation and burning of lipids, other than in glucose homeostasis, respiration, in morphogenesis and inflammatory response (Janani and Ranjitha Kumari, 2015). Desaturation and elongation also can take place in liver of fish with a pathway similar to the other vertebrates (Tocher, 2003). EPA and DHA biosynthesis pathway starts with the essential FA C18:3n-3 and involves fad_s and $elovl_s$, working in turn until the production of C24:6n-3, that is finally β-oxidised to C22:6n-3, i.e., DHA (Tocher, 2003). Alternatively, it has been recently pointed out that the *fads2* enzyme exhibits also Δ4 activity in some fish species, but findings were not confirmed in salmonids (Oboh et al., 2017). Nonetheless, salmonids have a more pronounced capacity of producing EPA and DHA in comparison to marine fish species, and this capacity is modulated by diets, among other factors (Turchini et al., 2009).

In the present study, an interdisciplinary approach was undertaken to explore the effects of dietary full-fat H meal in rainbow trout (*Oncorhynchus mykiss*), with particular concern on applied implications. Qualitative traits of rainbow trout fillets were closely analysed. In addition, liver FA composition as well as the expression of genes related to lipid metabolism in liver, pyloric caeca and mid intestine were investigated to question the biological implications of feeding a commercially important aquacultured species with the innovative protein source.

2. Materials and methods

2.1. Ethical statement, diets, fish rearing and tissue sampling

The fish feeding trial was performed at the experimental facility of the Agricultural, Food, Environmental and Animal Sciences Department of the University of Udine (Italy) as previously described by Cardinaletti et al. (2019). All procedure for animal handling and care were accomplished according to the guidelines of the European Union (Directive 2010/63/EU, 2010) and Italian law (D.L. 116/92) and the experimental protocol was approved by the Ethical Committee of the University of Udine (Prot. N. 1/2018).

Three isoproteic (40 g/100 g crude protein), isolipidic (18.5 g/100 g) and isoenergetic (22 MJ/kg) diets were prepared: one control diet was based on fishmeal and purified protein-rich vegetable ingredients with 0% H inclusion (H0) and two experimental diets were obtained from the H0 formulation by graded inclusion levels of H meal at 25% (H25) and 50% (H50) in place of fishmeal as basis (Table 1). Each diet was assigned to three groups of 30 juvenile rainbow trout (*Oncorhynchus mykiss*) of 137.3 ± 10.5 g initial body weight. Specimens were randomly allocated to nine 1 m^3 square fiberglass tanks and fed over 98 days as described in Cardinaletti et al. (2019). At the end of feeding trial, fish were euthanised with MS-222 and their organs sampled as follows: from four fish per dietary treatment, liver (L), pyloric caeca (C) and mid intestine (M, corresponding to the tract immediately behind the anterior segment to the ileorectal valve) were immediately excised, put in individual plastic tubes, frozen in liquid nitrogen and stored at -80 °C for the gene expression analyses. Moreover, the liver, viscera and fillets of five fish per diet were assigned to biometrics measurements and chemical analyses, and ten fillets from ten other individual fish per diet were allocated to physical analyses. Five fish per diet were frozen for investigations on gut microbiota, object of another study.

2.2. Biometrics and physical analyses

At the end of the feeding trial, after a 10-hour fasting period, five fish per diet were subjected to stage 3 anaesthesia with MSS-222 (300 mg/L). Individual body mass, liver, viscera, fillet and fillet's skin–were weighed, and the values of some

parameters were utilised for assessing hepatosomatic (HSI) and viscerosomatic indices (VSI):

HSI = [liver weight/total body weight (g)]×100

VSI = [viscera weight/total body weight (g)]×100

The incidence of skin on fillet was calculated following the formula:

$$\text{Incidence of skin of fillets } (\%) = \frac{\text{skin weight, g}}{\text{fillet with skin weight, g}} \times 100.$$

Ten fish per diet were allocated to the physical analyses. The colour of the fillets was measured on triplicate positions (cranial, medial and caudal) on both sides with a CHROMA METER CR-200 (Konica Minolta, Singapore Japan) following the CIELab system (CIE, 1976) and recording L* (lightness), a* (redness index) and b* (yellowness index) parameters. Colour values were recorded and ΔE between samples was calculated according to the following formula: $\Delta E_{(\beta-\alpha)} = \left[\left(L^*_\beta - L^*_\alpha\right)^2 + \left(a^*_\beta - a^*_\alpha\right)^2 + \left(b^*_\beta - b^*_\alpha\right)^2\right]^{0.5}$, where α and β represent alternatively the mean colour values of H0, H25 and H50.

The values of pH and water holding capacity (WHC) parameters were considered. The pH value was measured on triplicate fillet positions (cranial, medial and caudal) by a pH-meter SevenGo SG2™ (Mettler-Toledo, Schwerzenbach, Switzerland). Afterwards, fillets were skinned, homogenised and used to determine WHC (Iaconisi et al., 2018) and chemical composition, as described below.

Skin thickness was measured by a manual calliper (Salmoiraghi, Milano, Italy) in three positions (cranial, medial and caudal).

2.3. Chemical analyses on fillets and liver

Five fillets and five livers per diet were analysed for total lipid contents as well as fatty acid profile, following the methods described by Folch et al. (1957) and Secci et al. (2018), respectively. Minor modifications to the lipid extraction procedure were used to analyse the skin, specifically, the skin was left in the chloroform:methanol (2:1 v/v) solution for one hour on an orbital shaker instead of homogenising it with a laboratory mixer.

The products:precursors ratio was used to assess the desaturasing and elongating activities, as proposed by Renaville et al. (2013).

2.4. Nutritional indices and TBARS of fillets

From the FA profile, the Atherogenicity index (AI) and Thrombogenicity index (TI) were calculated according to Ulbricht and Southgate (1991) and the

Hypocholesterolaemic/Hypercholesterolaemic FA ratio (h/H) according to Santos-Silva et al. (2002):

Atherogenicity index (AI):

$$\frac{C12{:}\,0 + (4 \times C14{:}\,0) + C16{:}\,0}{MUFA + PUFAn\text{-}6 + PUFAn\text{-}3}$$

Thrombogenicity index (TI):

$$\frac{C14{:}\,0 + C16{:}\,0 + C18{:}\,0}{(0.5 \times MUFA) + (0.5 \times PUFAn\text{-}6) + (3 \times PUFAn\text{-}3) + (n\text{-}3/n\text{-}6)}$$

Hypocholesterolaemic/Hypercholesterolaemic FA ratio (h/H):

$$\frac{C18{:}\,1n\text{-}9 + C18{:}\,2n\text{-}6 + C18{:}\,3n\text{-}3 + C20{:}\,4n\text{-}6 + C20{:}\,5n\text{-}3 + C22{:}\,5n\text{-}3 + C22{:}\,6n\text{-}3}{C14{:}\,0 + C16{:}\,0}$$

n-3/n-6 ratio

The secondary lipid oxidation products (thiobarbituric acid reactive substances, TBARS) were evaluated on 2 g of homogenised samples according to Vyncke (1970).

2.5. Gene expression analyses

RNA extraction and cDNA synthesis

Total RNA extraction from liver, pyloric caeca and mid intestine samples from four different specimens per dietary treatment was optimized using RNAzol® RT reagent (Sigma-Aldrich®, R4533) following the manufacturer's instructions. Total RNA extracted was eluted in 20 µl of RNase-free water (Qiagen). Final RNA concentration was determined by the NanoPhotometer® P-Class (Implen, München, Germany). RNA integrity was verified by GelRed™ staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stored at -80 °C until use. Finally, 2 µg of RNA were used for cDNA synthesis, employing the High Capacity cDNA Reverse Transcription Kit (Bio-Rad, Milan, Italy) following the manufacturer's instructions.

Real-Time qPCR

Prior to qPCR reactions, all primer pairs were used in gradient reactions in order to determine the optimal annealing temperatures; control cDNA samples were pooled and used for this purpose. Then, PCR efficiency for each primer pair was determined using 10-fold serial dilutions of pooled liver, pyloric caeca and mid intestine cDNA samples, respectively.

qPCRs were performed with SYBR® Green in an iQ5 iCycler thermal cycler (both from Bio-Rad, CA, USA) in duplicate. For each sample, reactions (10 µL) were set on a 96-well plate by mixing 1 µL cDNA diluted 1:20, 5 µL of 2x concentrated SYBR® Green as the fluorescent intercalating agent, 0.2 µM forward primer, and 0.2 µM reverse primer. The thermal profile for all reactions was: 3

min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 20 s at a variable temperature depending on the primer annealing temperature (Table 2) and 20 s at 72 °C. Fluorescence was monitored at the end of each cycle. Dissociation curve analysis showed a single pick in all cases.

Relative quantification of the expression of genes involved in fish lipid metabolism (*fads2*, *elovl1*, *elovl2*, *pparα*, *pparβ*, *pparδ*, *pparγ* and *cd36*), was performed using *arp* and *60S* as housekeeping genes to standardize the results. The primer sequences were designed using Primer-BLAST tool available in NCBI (http://www.ncbi.nlm.nih.gov/). Data were analysed using the iQ5 optical system software version 2.0, including Genex Macro iQ5 Conversion and Genex Macro iQ5 files (Bio-Rad, CA, USA). Modification of gene expression was reported with respect to controls.

2.6. Statistical analysis

Data of biometrics, physical traits, chemical composition, nutritional indices and TBARS content were assessed for normality (Shapiro-Wilk test) and homoscedasticity (Levene's test). If normality and homoscedasticity were not met, a boxcox transformation was performed. Then, a one-way ANOVA followed by a Tukey's test were performed using the free software environment R (R Core Team, 2018), significance set at $p \leq 0.05$). Results are presented as means and pooled standard error of the mean (SEM), if not otherwise stated.

The statistical software package Prism6 (GraphPad Software, La Jolla, California, USA, www.graphpad.com) was used to analyse q-PCR data. Significance was set at $p \leq 0.05$ and all results are presented as means ± standard deviation.

3. Results

3.1. Biometrics measurements and physical traits of fillets

Biometrics measurements did not show significant differences between dietary treatments. HSI lined up at 1.08, 1.04 and 1.13%, while VSI was 9.81, 11.12 and 10.95% for Ho, H25 and H50 groups, respectively.

3.2. Physical traits and chemical composition of fillets

Fillets exhibited similar physical traits between dietary treatment (Table 3). The yellowness b* index was the only significantly different parameter, touching the lowest value in H25 fillets and the highest one in Ho fillets ($p<0.05$). ΔE calculations showed the following results: Ho *vs* H25: 2.28, Ho *vs* H50: 1.23, H25 *vs* H50: 1.37.

As shown in Table 4, total lipids and FA profile of fillets were not significantly affected by the different dietary regime, except for SFA. Total SFA, C12:0 and C14:0 increased with the increasing dietary inclusion of H meal ($p<0.01$, $p<0.001$, $p<0.01$, respectively), while C16:0 showed an opposite trend ($p<0.01$). C18:1n-9 was not significantly different between the groups, although values showed a decreasing trend following the increasing dietary H meal inclusion. In comparison to the composition of the diets, C18:2n-6 showed a mean value of 11.41 g FAME/100 g total FAME between groups. EPA+DHA was in average 14.92 g FAME/100 g total FAME. Products:precursors ratios showed that C18:4n-3/C18:3n-3 ratio was higher in H25 and H50 in comparison to H0 group.

3.3. Nutritional indices and TBARS of fillets

As displayed in Table 5, TI and n-3/n-6 ratio were not significantly different between dietary treatments, while the AI was higher in H50 in comparison to H0 and H25 groups ($p<0.001$), while the h/H ratio showed the highest value in H25 and was significantly lower in H50 ($p<0.05$). As shown in Figure 1, the levels of malondialdehyde-equivalents of the fillets belonging to groups H25 and H50 presented significantly lower values in comparison to H0 fillets.

3.4. Chemical composition of livers

Hepatic total lipid content was not significantly different between the dietary treatments, while FA profile seemed to be deeply affected (Table 6), resembling the FA profile of the diets and of the fillets. SFA were abundantly present in all the dietary groups (47.25 g FAME/100 g total FAME). C18:2n-6 was the highest in the H25 group and the lowest in the H0 group ($p<0.01$). The 20-carbon atom-long FAs all showed a decreasing trend following the increase in dietary H meal, except for EPA, whose value in the H50 group was significantly different from the two other groups ($p<0.001$), although the absolute difference was subtle. DHA did not significantly differ between groups and showed a mean value of 11.07 g FAME/100 g total FAME. Overall, SFA were not significantly different between the three groups whilst both MUFA and PUFAn-6 increased with the increasing inclusion of H meal ($p<0.05$ and $p<0.01$, respectively), and PUFAn-3 tended to show an opposite trend. The n-3/n-6 ratio was significantly lower in the two groups fed diets with H meal inclusion ($p<0.001$).

The products:precursors ratios did not highlight any statistically significant difference. Noticeably, DHA:C18:3n-3 ratio was high in all treatments (32.93, 32.65 and 40.53 in H0, H25 and H50, respectively).

3.5. Gene expression

The expression of *cd36*, *ppar*$_s$ and of those genes related to LC PUFA biosynthetic pathways was analysed (Figure 2a-h). In the pyloric caeca, *cd36* gene expression increased along with the dietary H meal inclusion, although not significant differences were detected ($p>0.05$). In the liver, the *ppar* gene (*ppara*, *pparδ* and *pparβ* and *pparγ*) expressions were not affected by the dietary

treatment; nonetheless, some decreasing trends following the H meal inclusion might be observed. The most interesting results were detected in the pyloric caeca, in which *fads2* gene expression increased in fish fed diets with increasing H meal level (Ho *vs* H50: $p<0.05$). A similar trend, despite not significant ($p>0.05$), was also observed in the mid intestine. Finally, no significant differences in the expression of *elovl1* and *elovl2* were observed in the different gastrointestinal tracts considered (L, C and M).

4. Discussion

The main results of the present study are encouraging and partly contradict some earlier findings, showing some unexpected and promising results. Results on biometrics measurements aligned with most of the results on rainbow trout fed with insect meals from different origins and at different dietary inclusion levels (Iaconisi et al., 2018; Renna et al., 2017; Stadtlander et al., 2017), even if some studies found a smaller HSI in rainbow trout fed with *Tenebrio molitor* or H meal, in comparison to fishmeal-based control diets, but the authors did not give an explanation of this observation (Belforti et al., 2015; Sealey et al., 2011). Also results on fillet physical traits found in the present study aligned with most of the results from similar studies (Iaconisi et al., 2018; Mancini et al., 2017). Colour is difficult to interpret as it depends on the presence of dietary pigments (especially, vegetable pigments), on rancidity in the case of the stored products, on the punctual readings of the colourimeter. Therefore, the increased yellowness index (b*) of H25 fillets is difficult to explain unambiguously. Possibly, the dietary palm oil had a role in fillet colour development. The ΔE result worth considering is the couple Ho *vs* H25, which was 2.28. This value is considered just above the threshold enabling unexperienced observers noticing colour differences (Mokrzycki and Tatol, 2011). Nevertheless, if one follows Sharma (2003)'s thresholds, a $\Delta E=2.28$ is below the limit enabling an unexperienced observer noticing the differences. Presumedly, a test with trained panelists could cast light on this cause.

Unexpected results originated from fillet FA content. In fact, only SFA showed an increasing trend parallelly to the increasing dietary inclusion of H meal, as commonly found in similar studies (Borgogno et al., 2017; Iaconisi et al., 2018; Stadtlander et al., 2017). On the other hand, not only did PUFAn-3 not show significant differences between the three dietary groups, but also the difference from the lowest (H50) to the highest (H25) value was 1.79 g of FAME/100 g total FAME. Also considering that fillets contained 4.81 g of total lipids/100 g fillet, the difference in PUFAn-3 between the three groups could be considered negligible. DHA reflected the trend found in the PUFAn-3 class. Furthermore, although C18:2n-6 was not present in the diets, the animals of the present study accumulated this FA in the fillets. The study of products:precursors ratios did not highlight any strong difference between dietary treatment, except for C18:4n-

3/C18:3n-3 ratio, that showed to be the lowest in Ho in comparison to H25 and H50 fillets.

The nutritional and freshness values of the fillets were assessed calculating AI, TI, h/H, n-3/n-6 ratios and TBARS content. The AI values in H50 were significantly higher than Ho and H25 groups, in accordance with Secci et al. (2019) but differently from Belforti et al. (2015) and Iaconisi et al. (2017). However, in the latter studies rainbow trout were fed with diets containing mealworm (*Tenebrio molitor*), which holds a large amount of MUFA, that passed to trout fillets. The TI values found in our study were not negatively altered, probably thanks to the relatively stable PUFAn-3 content of fillets. Conversely, TI rose in fillets of trout fed diets containing either mealworm or partially defatted H meal (Belforti et al., 2015; Iaconisi et al., 2017; Secci et al., 2019). In the present study, h/H pattern reflected the content of PUFAn-3 and DHA in the fillets, with the highest value highlighted in H25 and the lowest in H50. No negative effects at the variation of dietary mealworm inclusion was found in rainbow trout fillets (Iaconisi et al., 2018), while Secci et al. (2019) reported a significant decrease in trout fillets fed with partially defatted H meal. Lastly, our n-3/n-6 ratio remained rather stable between the treatments, unanimously with Iaconisi et al. (2018) but differently from Belforti et al. (2015) and Secci et al. (2019). A limited number of studies analysed the TBARS content in fish fed diets containing insect. Secci et al. (2019) found a decreasing but not significant trend in the fillets of rainbow trout fed diets where 0, 25 and 50% of the fishmeal had been replaced by partially defatted H meal. Similarly, our results highlighted that H-fed fish fillets contained a lower amount of TBARS. This finding could be attributed to the higher SFA and lower PUFA content in the fillets belonging to H25 and H50 groups.

Total lipids and FA profile of the livers approximately reflected the findings on fillets, with the difference that livers exhibited significant differences where fillets only showed a trend; also, total SFA in liver were not specifically different between the dietary groups, probably because the increase of C12:0 and C14:0 in H25 and H50 groups was counterbalanced by a decrease in C16:0. Belghit et al. (2018b) noticed a decrease in liver triacylglycerols content when freshwater A. salmon was fed diets containing H derivatives in comparison to the control diet, in addition to a low content in C12:0 regardless of the administered diet. In the present study, these findings were not met but, on the contrary, total lipid content was similar between the dietary groups and C12:0 content increased with the presence of H meal; it can be thus assumed that C12:0 was accumulated rather than being used for oxidation.

As concerns FA of the n-6 series, they mainly derived from endogenous production as they could be found in very little amounts in the experimental diets. DHA, tended to decrease in H25 and H50 diets, but its presence in liver as well as fillet was considerable. Also the DHA:C18:3 n-3 ratio was high in liver and fillet, suggesting either that liver accumulated DHA, or that elongase and desaturase enzymes synthesised this FA.

The effect of full-fat H meal administration on FA of rainbow trout, discussed from a macroscopic point of view, are now further discussed from the perspective of gene expression.

A weak increasing trend in pyloric caeca *cd36* expression was noted upon the increase of dietary H meal inclusion, while no manifest differences between dietary groups could be highlighted in liver and mid intestine. In a similar manner to the present study, Li et al. (2019) described an increased expression of *cd36* in the proximal intestine of pre-smolt A. salmon fed a diet where 85% of the control protein sources had been replaced by H meal, simultaneously raising dietary fish oil content and diminishing rapeseed oil. One must remember that *cd36* is a multifunctional receptor binding ligands involved in angiogenesis, inflammatory response, taste and other functions (The UniProt Consortium, 2019) and the function of this cluster of differentiation in rainbow trout has not been deeply studied. Eventually, the observed results cannot be directly attributed to H meal, on the other hand, the interaction with the other ingredients could have played a major role in *cd36* modulation.

It is accepted that the expression of *ppar*$_s$ changes depending on the dietary lipid quantity and quality (Tocher, 2003), in addition to a tissue-specific modulation (Morash et al., 2009). In the present study, mid intestine *pparα*, liver *pparδ* and the *pparβ* of the three tissues showed a faint decreasing trend following the increase of dietary H meal inclusion, while *pparγ* seemed to be moderately stable across the three different dietary regimes. Notwithstanding, no statistical difference was found and interindividual variability was high, so it was difficult to formulate sound hypotheses on the dietary effect on *ppar* genes expression. In addition, only the study by Belghit et al. (2018b) analysed the effect of H meal and two H oils on A. salmon *pparα* and *pparγ*, finding that *pparα* was not affected by the different diets and that *pparγ* was downregulated in the diets containing H meal (irrespective of the dietary oily source) in comparison to a diet containing protein from plant and marine sources and presenting a substitution of the rapeseed oil with the H oil. All the diets contained the same amount of LC PUFA, but the diets with H meal contained a slightly higher amount of total lipids than that of the diets lacking H meal (Belghit et al., 2018b). This element might possibly have contributed to the modulation of *ppar*$_s$ in a different way than that of the present study, as they are regulated by both the FA type and their overall quantity (Tocher, 2003).

Because of the fact that different diets bring qualitatively different FAs, *in vivo* studies are difficult to interpret. Feeding rainbow trout with a high SFA-diet increased the expression of liver *pparα* and *pparβ*, in comparison to a group fed with a high PUFA-diet (Morash et al., 2009). Rimoldi et al. (2016) concluded that European sea bass *pparα* and *pparβ* were upregulated during fasting, thus promoting fat burning, while *pparγ*, which usually promotes fat deposition, was downregulated. In an *in vitro* study on rainbow trout hepatocytes cultured with either of 12 different FAs up to 24 hours, *pparα* expression was upregulated by C18:3n-3, C20:4n-6 and DHA, while it was downregulated by C18:2n-6 and EPA

(Coccia et al., 2014); SFAs and MUFAs tended to upregulate *ppara*. Jointly considering the high interindividual variability and the fact that *ppar*$_s$ regulate a number of signalling pathways (Kortner et al., 2013), further research on *ppar*$_s$ is needed to understand their regulation and role in lipid metabolism.

It is widely accepted that elongase and desaturase gene expression is modulated by the dietary lipids; for instance, these genes are upregulated when fish are fed vegetable oil-based diets, possibly for the deprivation of LC PUFA or the increased content of C18:3n-3 and, consequently, PUFA production is stimulated in salmonids, as reviewed by Tocher (2015). In detail, high EPA and DHA is thought to depress desaturase expression in salmon A. salmon cell line (Zheng et al., 2009), dietary vegetable oil tended to upregulate *fads2* rainbow trout liver expression (Dong et al., 2017) and the factor blamed to increase *fads2* transcript in the liver of rainbow trout fed a plant-based diet was the high C18:3n-3 and the low LC PUFA contents in the diet (Véron et al., 2016). Even if LC PUFA production is stimulated, the EPA and DHA content in fish fed vegetable oil-based diets is not equal to that of fish fed fish oil (Tocher, 2015; Véron et al., 2016). Partly contradicting previous results, zebrafish larvae after 21 days post hatch and after six months of feeding with diets where 50% of fishmeal had been replaced by full-fat H meal showed an increased *elovl2* gene expression in comparison to the 25% substitution-group and the control group; *fads2* gene expression was uncertain in zebrafish larvae, but after six months feeding it was significantly higher in the maximum-replacement level group (Zarantoniello et al., 2019, 2018). The same authors also found a significantly decrease of zebrafish EPA after six months, but DHA content in the control and 25% replacement groups were even and significantly higher than in the 50% replacement group. Surprisingly, in the present study, it seemed clear that the dietary FA profile was changed by endogenous desaturase and elongase enzymes, as fillet DHA content did not reflect dietary content. Trends of mid intestine *elovl1* and liver *elovl1* and *elovl2* seemed declining, while pyloric caeca *elovl2* tended to increase at the increasing inclusion of dietary H meal. An increasing *fads2* expression was noted in pyloric caeca and mid intestine districts following the increasing dietary H meal inclusion, while the expression seemed stable in the liver. Partly explaining the biology of our findings, one must remember that pyloric caeca are a significant site of DHA synthesis in rainbow trout (Bell et al., 2003). The investigation on products:precursors ratios hinted that a higher deposition of C18:4n-3 in fillets of fish fed H meal was realised. A direct enzymatic activity assessment would be a useful tool to evaluate the real outcome of gene expression and to explain the endogenous mechanisms of PUFA biosynthesis.

5. Conclusions

The present study showed that full-fat H meal slightly affected rainbow trout qualitative traits, while guaranteeing a nutritious final product. Further investigations hinted that lipid metabolism played a role in ensuring a desirable

final eating quality. Future studies on the biological mechanisms behind the macroscopic traits of fish fed insects are warmly encouraged.

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Table 1. Ingredients (g/kg), proximate composition (g 100/g), total lipids (g/100g) and fatty acid profile (as percentage of total FAMEs) of the experimental diets.

	H0	**H25**	**H50**
Ingredients			
Chile prime fish meal[1]	420	315	210
Protein-rich vegetable ingredients[2]	110	156	200
H meal[3]	-	105	210
Wheat flour[4]	290	268	255
Fish oil	70	40	28
Palm oil	70	75	56
Mineral[$] and Vitamin[#] supplements	20	20	20
Binder	20	20	20
L-Methionine	-	1	1
Proximate composition[*]			
Moisture	4.24±0.03	5.49±0.03	5.31±0.18
Crude protein, CP	40.27±0.45	39.98±0.37	40.16±0.39
Ether extract, EE	18.63±0.27	18.56±0.14	17.68±0.20
Ash	14.30±0.28	14.20±0.23	14.13±0.31
Gross energy (MJ kg^{-1})	22.10±0.11	22.30±0.03	21.28±0.06
Total lipids	19.76±0.09	18.94±0.27	19.08±0.51
Fatty acid[*]			
C10:0	tr	0.36±0.03	0.74±0.04
C12:0	0.12±0.05	6.49±0.68	13.05±1.37
C14:0	2.89±0.16	4.02±0.31	5.68±1.05
C15:0	0.42±0.09	0.43±0.08	0.54±0.26
C16:0	23.63±1.63	24.11±2.81	21.62±1.21
C16:1n-9	0.11±0.04	0.13±0.05	0.18±0.02
C16:1n-7	3.24±0.36	3.88±0.91	5.25±0.84
C17:0	0.47±0.11	0.48±0.04	0.59±0.13
C18:0	5.57±0.74	5.44±1.11	5.08±0.23
C18:1n-9	26.25±0.55	27.85±1.65	24.97±1.76
C18:1n-7	1.29±0.38	0.99±0.16	0.98±0.31
C18:2n-6	0.11±0.03	tr	tr
C18:3n-3	1.59±0.13	1.13±0.23	0.95±0.35
C20:0	0.38±0.07	0.42±0.14	0.45±0.07
C20:1n-9	0.95±0.35	0.86±0.22	0.94±0.20
C20:2n-6	0.17±0.01	0.10±0.001	tr
C20:3n-6	0.12±0.04	tr	tr
C20:4n-6, ARA	0.73±0.74	0.49±0.13	0.41±0.06
C20:3n-3	0.15±0.07	0.10±0.14	tr
C20:5n-3, EPA	6.85±0.24	4.24±0.49	3.12±0.74
C22:0	0.19±0.03	0.27±0.01	0.36±0.07
C22:1n-9	0.56±0.13	0.39±0.05	0.35±0.08
C22:6n-3, DHA	13.42±0.89	8.24±0.36	5.97±0.72
C24:1n-9	0.47±0.38	0.37±0.08	0.38±0.08
SFA	33.76±0.14	42.13±4.51	48.24±1.67
MUFA	32.87±1.23	34.46±0.61	33.04±1.45
PUFAn-3	22.01±0.45	13.71±0.22	10.11±0.33
PUFAn-6	11.33±2.20	9.68±1.56	8.62±1.50
PUFAn-9	26.50±0.71	28.00±1.41	25.50±2.12
n-3/n-6	1.99±0.43	1.44±0.25	1.19±0.17

[1]Bioceval GmbH & Co. KG Cuxhaven, Germany.

[2] Protein-rich vegetable ingredients: blend of pea protein concentrate (Lombarda trading srl, Casalbuttano & Uniti (CR, Italy)) and wheat gluten meal (Sacchetto spa (Torino, Italy)) in 1:1 ratio.

[3]SmartBugs srl (Treviso, Italy).

[4]Consorzio Agrario (Pordenone, Italy).

[$]Mineral supplement composition (% mix): $CaHPO_4 \times 2H_2O$, 78.9; MgO, 2.725; KCl, 0.005; NaCl, 17.65; $FeCO_3$, 0.335; $ZnSO_4 \times H_2O$, 0.197; $MnSO_4 \times H_2O$, 0.094; $CuSO_4 \times 5H_2O$, 0.027; Na_2SeO_3, 0.067.

#Vitamin supplement composition (% mix): thiamine HCL Vit B1, 0.16; riboflavin Vit B2, 0.39; pyridoxine HCL Vit B6, 0.21; cyanocobalamine B12, 0.21; niacin Vit PP, 2.12; calcium pantotenate, 0.63; folic acid, 0.10; biotin Vit H, 1.05; myoinositol, 3.15; stay C Roche, 4.51; tocoferol Vit E, 3.15; menadione Vit K3, 0.24; Vit A (2500 UI kg^{-1} diet), 0.026; Vit D3 (2400 UI kg^{-1} diet), 0.05; choline chloride, 83.99.

**Values reported as mean of triplicate analyses.*

tr: fatty acids below of 0.1% of total FAMEs

SFA = C10:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C24:0.

MUFA = C16:1n-9 + C16:1n-7 + C18:1n-9 + C18:1n-7 + C20:1n-9 + C22:1n-9 + C24:1n-9.

PUFAn-3 = C18:3n-3 + C20:3n-3 + C20:5n-3 + C22:6n-3.

PUFAn-6 = C18:2n-6 + C18:3n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6.

Table 2. Primer pair sequences and annealing temperature conditions for genes used for real-time PCR.

Gene	3′ primer sequence		Annealing temperature (°C)
	Forward	Reverse	
fads2	GCCCTACCATCACCAACACC	AAACTCATCGACCACGCCAG	60.0
elovl1	TTGCCCAAGCAGGATACCAA	ATTCATGCGTCTTGGGTGTTC	59.2
elovl2	TGGATGGGTCCCAGAGATGA	AGAAGGACAAGATCGTGAGGC	56.0
pparα	AGTCGAGTAACGGCTCTGAAGG	CCGACACTCCAGGTTGAGAGA	60.0
pparβ	ATCAGCAGGAGAAGGGGAGTAG	GGAGACGATGTCTGGGACAGAT	58.2
pparδ	TCCTGTTTCCTGTGAGTGGGA	CCAGTCAGCACATTGCCATTTC	56.0
pparγ	GCCCTTATCGCCTTCTCAGT	AGAGCTGGCGTCTGTGTAAG	56.0
cd36	TCAAGCGTTGTCTGTAGTGAGT	CCCAGTAGCGTAAATTGCACA	58.2
arp	GAAAATCATCCAATTGCTGGA	CTTCCCACGCAAGGACAGA	60.0
60S	AGCCACCAGTATGCTAACCAG	TGTGATTGCACATTGACAAAA	60.0

Table 3. Physical parameters of fillets of rainbow trout fed the three experimental diets at increasing levels of full-fat *Hermetia illucens* larva meal (H0, 0%H; H25, 25%H; H50, 50%H).

	Diet			SEM[1]	*p-value*[2]
	H0	**H25**	**H50**		
pH	6.59	6.67	6.59	0.02	*ns*
WHC, %	91.66	93.89	91.82	0.52	*ns*
Colour					
L*	48.42	46.79	47.61	0.43	*ns*
a*	0.31	1.08	0.32	0.18	*ns*
b*	5.57^{a}	4.21^{b}	4.43^{ab}	0.21	*

[1]SEM: Standard error of the mean

[2]ns: not significant (p>0.05); a, b as superscript letters indicate significantly different means at p<0.05 (*).

Table 4. Total lipids (g/100 g fillet), fatty acid profile (g of FAME/100 g total FAME) and products:precursors ratio of fillets of rainbow trout fed the three experimental diets at increasing levels of full-fat *Hermetia illucens* larva meal (H0, 0%H; H25, 25%H; H50, 50%H).

	Diet			SEM[1]	*p-value*[2]
	H0	**H25**	**H50**		
Total lipids	5.13	4.42	4.87	0.36	*ns*
C12:0	0.26^{c}	2.15^{b}	5.00^{a}	0.57	***
C13:0	7.23	8.52	9.27	0.86	*ns*
C14:0	1.68^{b}	1.94^{b}	2.66^{a}	0.14	**
C16:0	15.47^{a}	13.51^{b}	13.89^{b}	0.30	**
C16:1n-7	2.13	2.10	2.15	0.08	*ns*
C18:0	3.59	3.44	3.49	0.05	*ns*
C18:1n-9	25.35	24.69	23.79	0.83	*ns*
C18:1n-7	2.32	2.16	1.88	0.10	*ns*
C18:2n-6	11.79	11.69	10.77	0.45	*ns*
C18:3n-3	2.07	1.85	1.66	0.08	*ns*
C20:1n-11	1.45^{a}	1.24^{ab}	1.15^{b}	0.05	*
C20:4n-6, ARA	0.85	1.11	1.10	0.05	*p=0.0568*
C20:5n-3, EPA	2.58	2.38	2.19	0.09	*ns*
C22:6n-3, DHA	12.70	13.03	11.87	0.71	*ns*
SFA	28.46^{b}	30.44^{b}	35.09^{a}	0.90	**
MUFA	29.77	30.95	29.66	1.01	*ns*
PUFAn-6	13.11	14.50	13.61	0.41	*ns*
PUFAn-3	18.48	19.76	17.97	0.75	*ns*
EPA+DHA	15.28	15.41	14.06	0.77	*ns*
SFA/UFA[3]	0.45^{b}	0.47^{ab}	0.58^{a}	0.02	*
C18:4n-3/C18:3n-3	0.21^{b}	0.29^{a}	0.29^{a}	0.01	**
C20:5n-3/C18:3n-3	1.26	1.33	1.37	0.08	*ns*
C22:6n-3/C18:3n-3	6.25	7.50	7.43	0.64	*ns*
C20:4n-6/C18:2n-6	0.07	0.10	0.10	0.01	*ns*

The following FAs were used for calculating the classes of FAs but they are not listed because below 1% of total FAME: C14:1, C15:0, C16:2n-4, C17:0, C16:3n-4, C17:1, C16:4n-1, C18:2n-6 trans, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-3, C18:4n-1, C20:0, C20:1n-11, C20:1n-9, C20:2n-6, C20:3n-6, C20:3n-3, C20:4n-3, C22:0, C22:1n-11, C22:1n-9, C22:4n-6, C21:5n-3, C22:5n-3, C24:0, C24:1.

[1]SEM: Standard error of the mean

[2]ns: not significant (p>0.05); a, b, c as superscript letters indicate significantly different means at p<0.05 (*); p<0.01 (**); p<0.001 (***)

[3]UFA: Unsaturated fatty acids.

Table 5. Nutritional indices of the fillets of rainbow trout fed the three experimental diets at increasing levels of full-fat *Hermetia illucens* larva meal (Ho, 0%H; H25, 25%H; H50, 50%H).

	Diet			SEM[1]	*p-value*[2]
	Ho	**H25**	**H50**		
AI	0.34[b]	0.36[b]	0.48[a]	0.019	***
TI	0.25	0.23	0.26	0.008	*ns*
h/H	3.30[ab]	3.63[a]	3.18[b]	0.080	*
n-3/n-6	1.41	1.42	1.35	0.089	*ns*

[1]SEM: Standard error of the mean

[2]ns: not significant (p>0.05); a, b as superscript letters indicate significantly different means at p≤0.05 (*); p≤0.001 (***).

Table 6. Total lipids (g/100 g liver), fatty acid profile (FAME/ total FAME) and products:precursors ratio of livers of rainbow trout fed the three experimental diets at increasing levels of full-fat *Hermetia illucens* larva meal (H0, 0%H; H25, 25%H; H50, 50%H).

	Diet			SEM[1]	*p-value*[2]
	H0	**H25**	**H50**		
Total lipids	11,79	10,53	11,66	0.34	*ns*
C12:0	0.13^{c}	1.41^{b}	3.16^{a}	0.24	***
C14:0	1.52^{c}	2.25^{b}	3.75^{a}	0.18	***
C16:0	32.99^{a}	29.09^{b}	25.81^{b}	1.02	**
C16:1n-7	1.28	1.52	1.51	0.05	*ns*
C18:0	14.10	13.02	13.65	0.24	*ns*
C18:1n-9	21.43^{b}	24.05^{a}	22.44^{ab}	0.48	*
C18:1n-7	2.00	2.08	1.97	0.04	*ns*
C18:2n-6	4.63^{b}	5.82^{a}	5.12^{ab}	0.16	**
C20:1n-9	2.33^{b}	2.86^{ab}	3.25^{a}	0.13	**
C20:2n-6	0.94^{b}	1.29^{ab}	1.42^{a}	0.07	**
C20:3n-6	0.56^{b}	0.93^{b}	1.04^{b}	0.05	***
C20:4n-6, ARA	1.44^{b}	1.88^{a}	1.86^{a}	0.07	**
C20:5n-3, EPA	1.15^{a}	0.94^{b}	0.73^{c}	0.04	***
C22:6n-3, DHA	11.55	11.26	10.41	0.29	*ns*
SFA	50.00	44.30	47.45	1.04	*ns*
MUFA	28.21^{b}	31.94^{a}	30.72^{ab}	0.68	*
PUFAn-6	7.79^{b}	10.15^{a}	9.66^{a}	0.31	**
PUFAn-3	13.62	13.27	11.89	0.35	*ns*
EPA+DHA	12.70	12.20	11.13	0.32	*ns*
SFA/UFA[3]	1.00	0.83	0.91	0.03	*ns*
n-3/n-6	1.78^{a}	1.32^{b}	1.23^{b}	0.06	***
C18:4n-3/C18:3n-3	0.36	0.39	0.34	0.05	*ns*
C20:5n-3/C18:3n-3	3.22	2.67	2.79	0.14	*ns*
C22:6n-3/C18:3n-3	32.93	32.65	40.53	2.06	*ns*
C20:4n-6/C18:2n-6	0.31	0.32	0.36	0.01	*ns*

The following FAs were used for calculating the classes of FAs but they are not listed because below 1% of total FAME: C14:1n-5, C15:0, C16:1n-9, C16:2n-4, C17:0, C16:3n-4, C17:1, C18:2n-4, C18:3n-6, C18:3n-4, C18:3n-3, C18:4n-3, C20:0, C20:1n-11, C20:1n-7, C20:3n-3, C20:4n-3, C22:0, C22:1n-11, C22:1n-9, C22:5n-3

[1]SEM: Standard error of the mean

[2]ns: not significant (p>0.05); a, b, c as superscript letters indicate significantly different means at p≤0.05 (*); p≤0.01 (**); p≤0.001 (***).

[3]UFA: Unsaturated fatty acids.

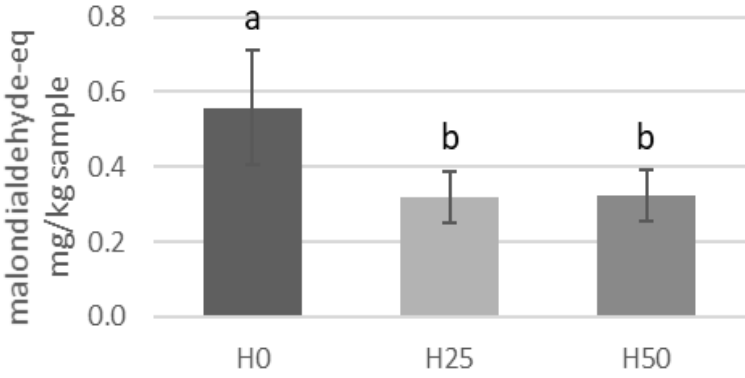

Figure 1. TBARS content of the fillets; values are expressed as mg MDA-eq/kg fillet.

Superscript letters indicate significant difference between groups ($p \leq 0.05$).

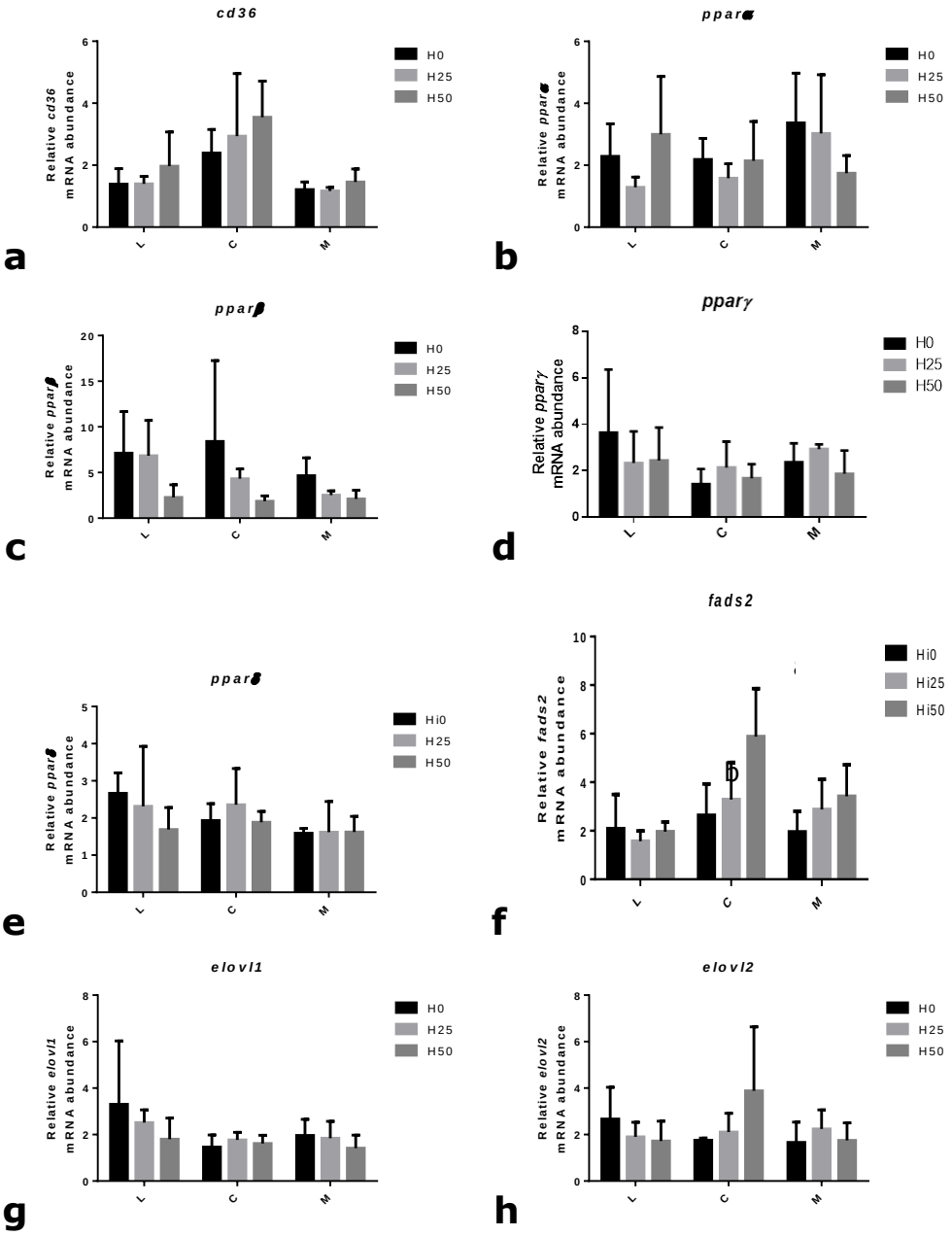

Figure 2. Relative mRNA abundances of genes related to the lipid metabolism. a: *cd36*; b to e: *ppar*$_s$; f: *fads2*; g and h: *elovl*.

Superscript letters indicate significant difference between groups ($p \leq 0.05$).

Table S1. Complete fatty acid profile (g of FAME/100 g total FAME) of fillets of rainbow trout fed the three experimental diets at increasing levels of full-fat *Hermetia illucens* larva meal (Ho, 0%H; H25, 25%H; H50, 50%H).

	Diet			SEM[1]	*p-value*[2]
	Ho	**H25**	**H50**		
C12:0	0.26	2.15	5.00	0.572	***
C13:0	7.23	8.52	9.27	0.862	*ns*
C14:0	1.68	1.94	2.66	0.136	**
C14:1	0.04	0.02	0.05	0.004	*
C15:0	0.19	0.18	0.17	0.005	*ns*
C16:0	15.47	13.51	13.89	0.296	**
C16:1n-7	2.13	2.10	2.15	0.075	*ns*
C16:2n-4	0.16	0.14	0.11	0.007	*
C17:0	0.24	0.21	0.20	0.005	*
C16:3n-4	0.08	0.07	0.05	0.004	**
C17:1	0.13	0.12	0.09	0.008	*ns*
C16:4n-1	0.13	0.11	0.10	0.004	**
C18:0	3.59	3.44	3.49	0.054	*ns*
C18:1n-9 cis	25.35	24.69	23.79	0.830	*ns*
C18:1n-7	2.32	2.16	1.88	0.099	*ns*
C18:2n-6 cis	11.79	11.69	10.77	0.453	*ns*
C18:2n-6 trans	0.13	0.11	0.10	0.004	**
C18:2n-4	0.21	0.33	0.24	0.018	**
C18:3n-6	0.05	0.05	0.05	0.002	*ns*
C18:3n-4	0.15	0.15	0.14	0.002	*ns*
C18:3n-3	2.07	1.85	1.66	0.078	*ns*
C18:4n-3	0.44	0.54	0.47	0.025	*ns*
C18:4n-1	0.12	0.12	0.12	0.003	*ns*
C20:0	0.19	0.13	0.12	0.010	***
C20:1n-11	1.45	1.24	1.15	0.052	*
C20:1n-9	0.10	0.10	0.09	0.003	*
C20:2n-6	0.81	0.84	0.88	0.022	*ns*
C20:3n-6	0.45	0.61	0.62	0.244	***
C20:4n-6	0.85	1.11	1.10	0.052	*ns*
C20:3n-3	0.21	0.16	0.13	0.009	***
C20:4n-3	0.45	0.34	0.29	0.019	***
C20:5n-3	2.58	2.38	2.19	0.086	*ns*
C22:0	0.40	0.29	0.23	0.021	***
C22:1n-11	0.05	0.05	0.05	0.002	*ns*
C22:1n-9	0.17	0.15	0.14	0.005	**
C22:4n-6	0.08	0.10	0.10	0.005	*ns*
C21:5n-3	0.28	0.39	0.39	0.023	*ns*
C22:5n-3	1.04	1.08	0.97	0.026	*ns*
C24:0	0.08	0.07	0.07	0.004	*ns*
C22:6n-3	12.70	13.03	11.87	0.713	*ns*
C24:1	0.35	0.33	0.28	0.017	*ns*
SFA	28.46	30.44	35.09	0.899	**
MUFA	29.77	30.95	29.66	1.014	*ns*
PUFAn-3	18.48	19.76	17.97	0.748	*ns*
PUFAn-6	13.11	14.50	13.61	0.413	*ns*

[1]SEM: Standard error of the mean

[2]ns: not significant (p>0.05); a, b, c as superscript letters indicate significantly different means at p≤0.05 (*); p≤0.01 (**); p≤0.001 (***).

Abstracts in Conference proceedings

- Li Y, **Bruni L**, Jaramillo-Torres A, Kortner TM, Chikwati EM, Belghit I, Lock EJ, Krogdahl Å (2019) Gut health and microbiota in post-smolt Atlantic salmon (*Salmo salar*) fed larvae meal from black soldier fly (*Hermetia illucens*). Aquaculture Europe 2019, Berlin (Germany)
- **Bruni L**, Secci G, Zarantoniello M, Randazzo B, Olivotto I, Tulli F, Parisi G (2019) Lipid metabolism and fillet quality of rainbow trout fed diets including *Hermetia illucens* full-fat larvae. 23rd Congress of the Animal Science and Production Association (ASPA) "New challenges in Animal Science", Sorrento (Italy)
- **Bruni L**, Secci G, Taiti C, Belghit I, Lock E-J, Parisi G (2019) Fillet fatty acids and volatile organic compounds of Atlantic salmon fed diets including *Hermetia illucens* larvae. 23rd Congress of the Animal Science and Production Association (ASPA) "New challenges in Animal Science", Sorrento (Italy)
- Secci G, **Bruni L**, Borgogno M, Taiti C, Belghit I, Lock E-J, Parisi G (2019) Fillet qualitative characteristics of Atlantic salmon fed diets including *Hermetia illucens* larvae. 23rd Congress of the Animal Science and Production Association (ASPA) "New challenges in Animal Science", Sorrento (Italy)
- Secci G, **Bruni L**, Parisi G (2019) Consumers' perception of insect meal as aquaculture feed ingredient. 23rd Congress of the Animal Science and Production Association (ASPA) "New challenges in Animal Science", Sorrento (Italy)
- **Bruni L**, Secci G, Faccenda F, Parisi G (2018) Effetto dell'introduzione di farina di alghe sugli aspetti merceologici e sulla qualità della trota iridea *Oncorhynchus mykiss*. 49° Congresso della Società Italiana di Biologia Marina, Cesenatico (Italy)
- **Bruni L**, Pastorelli R, Viti C, Gasco L, Basto A, Peres H, Parisi G (2017) Poster session: *Hermetia illucens* defatted larvae meal as partial dietary protein source for rainbow trout (*Oncorhynchus mykiss*): hepatic oxidative stress and intestinal microbial communities. 3rd international INSECTA Conference 2017, Berlin (Germany)
- Secci G, Bovera F, Loponte R, **Bruni L**, Panettieri V, Parisi G (2017) Total substitution of soybean protein source with *Hermetia illucens* meal in Lohmann Brown Classic hens diet: effect on liver and visceral fat composition. 3rd international INSECTA Conference 2017, Berlin (Germany)

Conclusion

The studies were conducted during a three-year PhD project and had the ambition to investigate some of the effects of feeding the Salmonidae Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) with diets containing *Hermetia illucens* larvae. Three topics were considered: gut microbiota, lipid metabolism and the final quality of fish.

Both studies on gut microbiota confirmed that microbiota composition was different between mucosa and digesta samples in A. salmon and rainbow trout fed diets containing *H. illucens* larvae meal; digesta samples tended to show higher biodiversity in comparison to mucosa samples.

In agreement with the literature, the gut bacterial community of A. salmon and rainbow trout was shaped by dietary interventions; specifically, feeding both Salmonids with diets containing *H. illucens* meal raised biodiversity.

Some specific bacterial *taxa* were found to be differentially modulated by either sample type (mucosa *vs* digesta) or diet factor. Some bacteria may protect fish from pathogens as well as enhance digestion, physiological functions and welfare in general; the effects that modified bacterial communities could have on fish biology must be examined.

Genotypic fingerprinting with denaturing gel electrophoresis technique proved to be a cheap and fast way to get an overview of the microbiota; moreover, by excising and sequencing selected bands, information down to the species-level can be obtained. However, this technique unveiled only a very restricted portion of the whole microbial community.

On the other hand, next generation sequencing technology was able to reveal the presence of a much larger amount of microorganisms. By high-throughput sequencing, the importance of analysing a mock community and extraction and library blanks was lifted, so that 1) the accuracy of the sequencing is verified and 2) contaminant sequences may be filtered out in the downstream bioinformatic pipeline.

Replacing up to 100% of fishmeal with *H. illucens* larvae meal produced minor effects on protein, fatty acid composition and fillet quality of A. salmon. The study demonstrated that it is possible to successfully maintain or even increase the cardioprotective characteristics of A. salmon fillets by modulating the oily ingredients and by tailoring *H. illucens* larvae by rearing them on a substrate from marine origin.

While no alteration in the volatile organic compounds of the fillets was noticed between different dietary groups, different cooking methods had an effect. Nonetheless, the consumer test on steamed fillets demonstrated that samples were evenly appreciated irrespective of the dietary group, although small weaknesses related to colour intensity and textural attributes were observed in the fillets of A. salmon fed the insect.

In rainbow trout, the fillet fatty acid profile of fish fed the full-fat *H. illucens* larvae meal only partly resembled the fatty acid profile of the diets. Indeed, the content of docosahexaenoic acid in rainbow trout fed full-fat *H. illucens* meal did not reflect the low dietary content of this fatty acid, and thus a nutritionally healthy food was guaranteed. Interestingly, no modulation of the dietary fish oil was operated.

Lipid metabolism was estimated in rainbow trout by computing the indices of fatty acid metabolism and the ratio of products:precursors, and by analysing gene expression by qPCR. The results produced with the three methods agreed on the fact that lipid metabolism was affected by the different diets.

The indices of fatty acid metabolism and the ratio of products:precursors were used to estimate the activity of desaturase and elongase enzymes. This approach allowed to get an overview on the enzyme activities, although these calculations are based on the fillet fatty acid composition and the risk is to restate the same findings of the fatty acid profile. Nonetheless, they can be useful for a rapid summary of the lipid metabolism.

The gene expression analyses partly corroborated these findings, as pyloric caeca *elovl2* tended to increase when rainbow trout were fed diets containing the full-fat insect. Also pyloric caeca and mid intestine *fads2* expression increased following the increasing dietary insect. On the other hand, mid intestine *pparα*, liver *pparδ* and the *pparβ* of pyloric caeca, mid intestine and liver showed a faint decreasing trend following the increase of dietary H meal inclusion. These results need reinforcement.

Outlook

The present thesis hopes to contribute with new knowledge regarding the effects of dietary *H. illucens* larvae meal on gut microbiota, lipid metabolism and final eating quality of rainbow trout and A. salmon. As the circle of knowledge expands, the following questions are raised:

What are the functional roles of bacteria? How does the interaction between host, guest and diet work? What are the biological consequences of this interaction? Does microbiota modifications have an effect on lipid metabolism? Combining the description of bacterial communities with omics technologies *as well as* old-fashioned culturing techniques seems highly desirable.

Likewise, combining different methods (fingerprinting techniques, next generation sequencings, culturing techniques and innovative techniques) may boost the understanding of this largely unknown dark matter.

How can eco-sustainability of aquaculture be fostered ensuring a nutritious final product when ingredient such as insects, which are poor in the cardioprotective fatty acids, are used? Increasing fish oil, however, undermines the sustainability assumption. Lipid metabolism showed to be affected, contributing to ameliorate the fatty acid profile. Deeper understanding of the dynamics behind lipid metabolism is needed.

Why was liver gene expression not significantly modulated despite the relevant role that this organ plays in lipid metabolism? Direct elongase and desaturase activity assessment would be a useful tool to evaluate the actual outcome of gene expression and to explain the endogenous mechanisms of fatty acid biosynthesis. Also, *in vivo* studies are difficult to interpret, thus cell culture could help to this cause.