


Application of laboratory methods for understanding fish responses to black soldier fly (*Hermetia illucens*) based diets

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Abstract

A major challenge for development of sustainable aquafeeds is its dependence on fish meal and fish oil. Replacement with more sustainable, nutritious and safe ingredients is now a priority. Over the last years, among several alternatives proposed, insects have received great attention as possible candidates. In particular, the black soldier fly (*Hermetia illucens*; BSF) represents a concrete example of how the circular economy concept can be applied to fish culture, providing a valuable biomass rich in fat and protein valorising organic by-products. In the last decade, several studies have been published about the use of different BSF dietary inclusion levels for various fish species including experimental models. Varying and encouraging results have been obtained in this research field using a plethora of laboratory methodological approaches that can be applied and coupled to obtain a comprehensive view of the BSF-based diets effects on fish physiology, health, and quality. The present review aims to explore some of the most promising laboratory approaches like histology, infrared spectroscopy, gut microbiome sequencing, molecular biology, fish fillets' physico-chemical and sensory properties, essential for a better understanding of fish welfare and fillet quality, when BSF is used as aquafeed ingredient. In particular, great importance has been given to European finfish species and experimental models.

Keywords: insect meal, alternative proteins, fish welfare, circular economy, sustainable aquaculture

1. Introduction

Due to the global capture fisheries stagnation, aquaculture is presently the fastest growing food production sector worldwide (FAO, 2018). Over the last years, there has been a significant increase in worldwide consumption of aquatic products (FAO, 2016) which is expected to further expand over the next thirty years (Gutiérrez *et al.*, 2020). In this context, the aquaculture industry will face an important challenge: providing a world population estimated to reach 9.7 billion by 2050 with a proper amount of nutritious and safe aquatic food (FAO, 2016; Godfray *et al.*, 2010). In order to meet this challenge, aquaculture production has to grow further (FAO, 2018). Although there is a high market demand for fish, a further development of aquaculture poses serious environmental challenges. As a result of ecological impacts of aquaculture inputs and resources, including

water, land and feed, the sustainability in aquaculture is often restricted by a wide range of environmental concerns (Ahmed and Thompson, 2019; Naylor *et al.*, 2005). Today, sustainable aquaculture technology and know-how have significantly advanced and they can play a pivotal role in achieving environmental sustainability goals. Long-term growth of the aquaculture industry has to be based on the concept of environmentally friendly practices and sustainable resource management. Presently, after the launch of Horizon 2020 by the European Community, aquaculture priorities can be summarised in three keywords: safe, sustainable, responsible. These words represent the basis for better animal's welfare, less environmental impact, and the development of new approaches able to provide larger volumes of healthy and safe food.

Aquaculture feed accounts for 50-70% of production costs (Van Huis *et al.*, 2013) and represent an equal share of the CO₂ footprint (Winther *et al.*, 2020). Because of the over-exploitation of pelagic fisheries, strategies to replace fish meal (FM) and fish oil (FO) in aquafeeds became both a private and public priority (Tacon and Metian, 2015). The study for low cost and sustainable feeding alternatives to totally or partially replace FM and FO have gained relevance (Henry *et al.*, 2015; Voorhees *et al.*, 2019) and to help the aquaculture industry to expand and remain competitive. Alternative ingredients are, for example vegetable proteins, including oilseeds (especially soybeans), and meat by-products (such as blood meal and bone meal). Among others, plant meals (PM) are the most widely used alternatives to FM since 2006 (Gatlin *et al.*, 2007; Gerile and Pirhonen, 2017; Hardy, 2010). However, PM have unbalanced essential amino acids profile (due to the deficiency in methionine and lysine), low protein content, anti-nutritional factors and a significant amount of non-digestible carbohydrates, which often limit their use for carnivorous fish (Basto-Silva *et al.*, 2019; Yasothai, 2016). In particular, non-digestible carbohydrates, including non-starch polysaccharides, may bind to bile acids or obstruct the action of digestive enzymes as well as feed transit in the intestine reducing both nutrients digestibility and absorption (Francis *et al.*, 2001). In addition, PM can cause inflammation of the digestive tract of fish (Blaufuss *et al.*, 2020; Henry *et al.*, 2015) but also show low palatability (Henry *et al.*, 2015; Papatryphon and Soares, 2001). Over the years, several techniques have been developed to partially overcome these issues including the use of enzymes, heat processes and the inclusion of bioactive compounds in the formulated diets (Gatlin *et al.*, 2007; Oliva-Teles *et al.*, 2015). In recent years, PM registered a significant rise in prices because of its increased use in human nutrition, causing a competition between the animal feed and human food sectors (Nogales-Mérida *et al.*, 2018). As a consequence, PM industry has limited potential to expand its production without putting additional pressure on arable land use and water consumption and shifting resource demand from oceans to land (Hua *et al.*, 2019).

Another alternative ingredient to FM in aquaculture is animal by-product meal (ABP). These meals contain a good balance of essential amino acids, show high protein content and good digestibility with features similar to those of FM (Barreto-Curiel *et al.*, 2016; EL-Haroun *et al.*, 2009; Hatlen *et al.*, 2015). Although the ABP seems to be a good and viable alternative, at technical and economic level, the consumer acceptance, a strict regulation and many restrictions for their use due to a lack of knowledge on the risk for developing human diseases are its main limitation (European Commission, 2013). In the European Union, the use of ABP was prohibited from 1990 to 2000 due to the arising of the bovine spongiform encephalopathy in ruminants and then, in 2013, was allowed only for

ABP derived from non-ruminant animals (Category 3) (Moutinho *et al.*, 2017). European legislation states that Category 3 ABP can be processed for feeding aquatic animals to contribute responsibly to both environment and public health (Gasco *et al.*, 2020).

Recently, the circular economy concept has gained great attention within the European Community and the EC Directive No. 2008/98 (EC, 2008), which establishes the order of importance in the choice of by-products treatment (the first being their recycle and the last being their landfill disposal) is playing a key role.

In order to meet the circular economy concept, aquafeed production can take advantage of the great amount of organic by-products which can be converted in a valuable biomass rich in proteins and lipids, by using bio-converting organisms (Lopes *et al.*, 2020; Parodi *et al.*, 2020; Truzzi *et al.*, 2020; Zarantoniello *et al.*, 2020b). Specifically, bioconversion through insects grown on organic by-products as feed source might represent a valuable solution (Barroso *et al.*, 2014; Belghit *et al.*, 2019a; Henry *et al.*, 2015). It also represents a valid example of sustainable animal production in terms of land use, water consumption and CO₂ production, because of the low energy requirements during rearing procedures (Berggren *et al.*, 2019; Smetana *et al.*, 2019). The nutritional values of the insects' biomass are generally characterised by high fat and protein content (Barroso *et al.*, 2014; Giannetto *et al.*, 2020); however, the insects' biomass nutritional composition depends on the quality and quantity of feed offered for their growth (Gobbi *et al.*, 2013; Pimentel *et al.*, 2017; Truzzi *et al.*, 2020; Zarantoniello *et al.*, 2020b), with the fat content and quality varying the most (from 7 to 39% dry matter) (Barragan-Fonseca *et al.*, 2017; Truzzi *et al.*, 2020).

Among the insects species used for aquafeed production, *Hermetia illucens* (L.) (Diptera, Stratiomyidae) (black soldier fly; BSF) is one of the most promising species: it shows high efficiency as bio-converter of organic by-product during the larval development (Salomone *et al.*, 2017), a proper protein content (up to 60% dry weight) and an essential amino acid pattern similar to that of FM (Müller *et al.*, 2017; Spranghers *et al.*, 2017). Furthermore, BSF larvae represent a good source of lipids usually dominated by saturated fatty acids (SFA) (especially rich in lauric acid, 12:0), a medium chain fatty acid which has anti-inflammatory and immune-boosting properties (Gasco *et al.*, 2018; Sealey *et al.*, 2011). Their high content in SFA rather than in polyunsaturated fatty acid (PUFA) still represent a key-issue to be solved since this unbalanced fatty acid (FA) profile could impair fish growth, welfare and quality (Ewald *et al.*, 2020).

Recently, methods to modulate the final biomass' nutritional profile by enriching the growing substrate have been developed (Liland *et al.*, 2017; St-Hilaire *et al.*, 2007). In

particular, Truzzi *et al.* (2020) demonstrated that growing BSF prepupae on a rearing substrate enriched with 10% *Schizochytrium* sp. can improve insect's final biomass, especially in terms of some FA like PUFA.

The inclusion of new ingredients in aquafeeds must be approached cautiously, since it is well-known that modulatory effects of different feed ingredients on fish physiological responses, quality and safety exist (Li *et al.*, 2019; Rimoldi *et al.*, 2019). The scientific community should take into consideration productivity, fish welfare and end-product's quality in order to guarantee both animals and humans wellbeing.

While productivity is not affected by proper insect meal inclusion levels in aquafeeds (Belforti *et al.*, 2015), the main problem found in the use of insect meal in fish production is changes in the FA profile of the fish product (Ferrer Llagostera *et al.*, 2019). When fed on insect meal based diets, fish fillets generally showed a reduced n-3/n-6 ratio, as well as low PUFA content, reducing the nutritional quality of fish for human consumption (Devic *et al.*, 2018; Ruxton *et al.*, 2004). In addition, since a large variety of organic by-products can be used as feed for insects, potentially toxic elements and pathogen microorganisms can enter the food production chain and be potentially harmful to animals and humans (Bosch *et al.*, 2019; Swinscoe *et al.*, 2019). Therefore, to meet a safe production of insects, a strict chemical and microbiological monitoring is necessary.

During the last decade, the issue of farmed fish welfare has raised increasing public and scientific concern (Panagiotaki and Malandrakis, 2019). Animal welfare has been defined as 'the aptitude of an animal to familiarise to its environment and maintain good health, while living a natural life and show its natural behaviour' (Ashley, 2007; Brijs *et al.*, 2018). Much research on the welfare of farmed fish is thus required to provide recommendations for best practices and future legislation (Ashley, 2007; EFSA, 2009). Farmed fish are usually exposed to a variety of stressors including handling, stocking density and nutrition which long-term exposure could have negative effects on health and growth performances (Barton, 2002; Olivotto *et al.*, 2002; Piccinetti *et al.*, 2015; Tocher, 2010). When it comes to new feed ingredients the gastrointestinal tract plays a key role. It constitutes an important barrier to the external environment (Groschwitz and Hogan, 2009), providing defence against pathogens and tolerance to dietary antigens (Peterson and Artis, 2014) while playing, at the same time, a fundamental role in the absorption of nutrients (Uran *et al.*, 2008) and on the innate and adaptive immunity of fish (Donaldson *et al.*, 2015; Kinnebrew and Pamer, 2012). Therefore, intestine integrity is considered to be essential to sustain a proper fish growth and welfare.

Presently, the fast development of science and the development of several new laboratory techniques allow scientist to have a deep and comprehensive approach when studying the effects of new practical diets in aquaculture. Numerous laboratory techniques like histology, infrared spectroscopy, gut microbiome sequencing, molecular biology as well as fish fillets' physico-chemical and sensory properties are now available for a better understanding of the above-mentioned aspects related to fish farming, representing an up-to date approach for a better understanding of the effects of insect-based diets on fish welfare and production.

The present review aims to provide an overview on the main analytical methods presently available for understanding welfare and quality of fish when fed on diets including BSF meal. Emphasis is given to a number of fish species of interest for the European aquaculture as well as to experimental models.

2. Fish gut and liver

Histology

Novel feed formulations for fish need to evaluate not only economical cost/benefit but also the effect on fish welfare. The gastrointestinal system is the primary target of dietary changes and challenges (Giorgini *et al.*, 2018a).

Histological analysis of the digestive apparatus is considered one the main approaches to evaluate fish welfare and nutritional status (Raskovic *et al.*, 2011). In particular, intestine and liver are the most important organs involved in digestive and immune functions and, consequently, are of particular interest when new ingredients are applied to aquafeed formulation (Ray and Ringø, 2014; Robaina *et al.*, 1995; Zhang *et al.*, 2020). Intestinal morphology is considered one of the main indicators of fish health since its morphological structure rapidly and often reversibly changes in response to dietary inputs. Alteration of gut integrity may modify nutrient absorption and thus fish welfare and growth, possibly affecting productivity (Krogdahl *et al.*, 2015, 2010; Penn *et al.*, 2011; Santigosa *et al.*, 2011; Voorhees *et al.*, 2019; Zhang *et al.*, 2013). Morphometric assessment of intestine architecture is widely used for studying intestine response to dietary challenges and involves the measurement of a number of histopathological parameters, in fish species (Baeverfjord and Krogdahl, 1996; Daprà *et al.*, 2011; Gu and Li, 2004; Krogdahl *et al.*, 2003; Swatson *et al.*, 2002).

Histological analysis relies on the use of tissue staining techniques to visualise intestinal morphology and specific cell markers. Among the most widely used stainings, haematoxylin and eosin staining (H&E) is commonly applied to provide general morphological information and

to detect and discriminate inflammatory cells (lymphocytes, granulocytes, melanomacrophages, etc.) (Mokhtar, 2017) based on their acidophilic (eosin) or basophilic (haematoxylin) features.

Other stainings allow to obtain more accurate information about the chemical composition of some cell categories, by virtue of specific chemical reactions (Dama and Pathan, 2019). As for example, periodic-acid Schiff (PAS) staining and Alcian blue pH2 (AB) staining are elective for neutral and acid (PAS and AB, respectively) mucins in mucous cells (Cardoso *et al.*, 2015; Purushothaman *et al.*, 2016).

The analysis of mucous cells in the intestinal epithelial layer is also of particular interest (Cardinaletti *et al.*, 2019; Zarantoniello *et al.*, 2021). These cells are able to produce and release defensive substances including mucins, lectins, toxins, immunoglobulins and antimicrobial peptides in response to specific dietary stimuli or mechanical injury (Bosi *et al.*, 2017; Hasnain *et al.*, 2013; Lazado and Caipang, 2014).

Aside providing information on the cell type and tissue morphology, histological analysis can be useful to obtain a number of histopathological parameters able to provide (semi-) quantitative information on possible intestinal alterations through a multi-grade scoring system (Silva *et al.*, 2015).

The most traditional histopathological indexes include: mucosal folds morphology (length, width, fusion), enterocytes supranuclear vacuolisation, *lamina propria* and submucosa width, leucocyte infiltration (also named 'cellularity'), and mucous cells abundance (Baeverfjord and Kroghdahl, 1996; Knudsen *et al.*, 2008; Laporte and Trushenski, 2012; Morris *et al.*, 2005; Penn *et al.*, 2011; Uran *et al.*, 2008). Since insect meal is known to include different molecules like chitin and short-medium FA, which may have an important role in gut welfare regulation (Bruni *et al.*, 2018; Gasco *et al.*, 2018; Rimoldi *et al.*, 2019), the analysis of all these parameters has recently been applied to several studies in order to provide information on possible inflammation and/or alterations in the nutrient transport in fish (Li *et al.*, 2020b).

Most of the histological studies have been performed on Atlantic salmon (*Salmo salar*) (Li *et al.*, 2019; Lock *et al.*, 2016), rainbow trout (*Oncorhynchus mykiss*) (Cardinaletti *et al.*, 2019; Renna *et al.*, 2017) and zebrafish (*Danio rerio*) (Zarantoniello *et al.*, 2019, 2020b), while a few studies are available on Siberian sturgeon (*Acipenser baerii*) (Caimi *et al.*, 2020a,b; Józefiak *et al.*, 2019b; Zarantoniello *et al.*, 2021) and Japanese sea bass (*Labrax japonicas*) (Wang *et al.*, 2019).

As regards Atlantic salmon, studies which adopted histological analyses in support to other laboratory techniques,

showed that a partial (40%) up to a total substitution of FM with full-fat (Lock *et al.*, 2016) or partially defatted (Li *et al.*, 2020b) BSF prepupae meal did not cause negative effects on post-smolt Atlantic salmon intestine morphology, indicating a high tolerance of salmonids to high dietary BSF meal inclusion levels.

The inclusion of a full-fat BSF prepupae meal in pre-smolt Atlantic salmon diet showed beneficial effects on fish intestine by reducing the enterocyte hyper-vacuolisation which usually characterises intestine of fish fed diets including high levels of soybean meal (Heikkinen *et al.*, 2006; Li *et al.*, 2019) and decreased steatosis in proximal intestine (Li *et al.*, 2020b).

Moreover, studies performed on rainbow trout showed that up to a 50% dietary substitution of FM with partially defatted (Dumas *et al.*, 2018; Renna *et al.*, 2017) BSF prepupae meal did not affect anterior or distal intestinal tracts morphology. However, Józefiak *et al.* (2019a) showed a slight decrease of villi height in trout proximal intestine fed a 50% dietary full-fat BSF inclusion level with respect to FM. In another recent study performed on rainbow trout fed diets containing 25 or 50% full-fat BSF meal with respect to FM (Cardinaletti *et al.*, 2019), no intestine severe inflammatory events were highlighted but a significant mucosal folds height reduction was observed in both groups, respected to control. In addition, Randazzo *et al.* (2020b, 2021b) showed a dose dependent increase of distal intestine mucous cells in rainbow trout fed diets in which 30 or 60% of vegetable proteins were replaced with defatted BSF prepupae meal. This result suggests a possible involvement of undigested chitin in inducing a higher lubrication of the intestine final tract. Similarly, it was demonstrated that dietary administration of full-fat BSF prepupae meal (25 or 50% with respect of FM) stimulated a higher secretion of neutral mucins rather than the acidic ones, along the entire digestive tract of rainbow trout (Cardinaletti *et al.*, 2019). These results suggest a possible involvement of undigested chitin in inducing a higher lubrication of the intestine final tract. Differently, Elia *et al.* (2018) did not evidence significant differences in mucous cells (both rich in neutral and acidic mucins) in rainbow trout, independently of partially defatted BSF larvae meal dietary inclusion (25 or 50%). However, the role of chitin in inducing mucous cells proliferation has still not been directly demonstrated and further studies are necessary.

To date, no studies are available on the effects of BSF meal dietary inclusion on European sea bass (*Dicentrarchus labrax*) intestinal histology, while one has recently been published on gilthead seabream (*Sparus aurata*) by Randazzo *et al.* (2021a). In addition, a recent study on Japanese sea bass showed that a replacement up to 64% of FM by defatted BSF meal did not affect intestine integrity (Wang *et al.*, 2019).

Only recently, a few studies focused on the effects of BSF prepupae meal dietary inclusion on Siberian sturgeon gut morphology: Józefiak *et al.* (2019b) showed that a diet in which 15% of FM was replaced by full-fat BSF meal caused a reduction in intestinal muscular and mucosal thickness but did not affect mucosal folds length, while Caimi *et al.* (2020a) did not report histological alterations of the distal intestine in fish fed highly defatted BSF meal (25 or 50% replacement with respect to FM).

Conversely, Zarantoniello *et al.* (2021), in juvenile sturgeons, demonstrated that a 50% dietary inclusion level of full-fat BSF meal with respect to FM induced mucosal folds atrophy and a dramatic decrease of enterocyte vacuolisation.

Recently, the effects of the administration of BSF-based diets in the experimental model zebrafish were deeply investigated. Particularly, a general increase in mucous cells number was observed in zebrafish larvae fed exclusively on full-fat BSF prepupae meal (Vargas *et al.*, 2018). On the contrary studies in which zebrafish were fed on diets including increasing inclusion levels of full-fat BSF prepupae meal (with respect to FM) did not show intestine morphology changes (Zarantoniello *et al.*, 2019, 2020a,b).

In addition to intestine, liver is often considered a second target organ when testing new aquafeed ingredients. This is particularly important when testing insect meal since its FA composition, rich in SFA, may alter the lipid accumulation in this organ (Vargas-Abúndez *et al.*, 2019). Liver welfare is a key aspect for fish production since this organ plays a central role in many of the fish metabolic pathways and its morphological structure and macromolecular composition are deeply influenced by the diet (Bruni *et al.*, 2020b; Cardinaletti *et al.*, 2019; Novriadi *et al.*, 2018; Vargas-Abúndez *et al.*, 2019).

Several histological analyses, using the above-mentioned histological stainings, may be applied to the liver, providing information about lipid accumulation, inflammation, necrosis, and glycogen deposition in this organ.

In 1997, McFadzen *et al.* (1997) proposed a criteria scale for the analysis of fish liver in order to determine the nutritional conditions and this approach has been successfully applied over the years for studying the effects of dietary challenges on farmed fish species (Sabbagh *et al.*, 2019). Liver histological parameters are responsive to food quality and availability (O'Connell, 1976) and are particularly addressed to provide information on hepatocyte morphology, with emphasis on the relative amount of intracellular vacuolisation associated with lipid and glycogen storage.

The fatty acid profile of insects does not always match the nutritional requirements of fish since insects are rich in

medium-chain SFA and MUFA rather than in long-chain PUFA. Liver lipid and glycogen deposition can provide important information on the effects and suitability of BSF-based diets (Bruni *et al.*, 2020b; Cardinaletti *et al.*, 2019; Li *et al.*, 2016). While traditional staining (H&E) allows to detect hepatocytes lipid storage, histochemical PAS staining helps in detecting liver glycogen accumulation (Aziza *et al.*, 2013; Bui-Nguyen *et al.*, 2015).

In post-smolt Atlantic salmon fed full-fat BSF prepupae meal diets (from 33 up to 100% with respect to FM), a dose-dependent increase in liver lipid accumulation was observed (Belghit *et al.*, 2019a), while only minor effects on liver lipid deposition were observed in Japanese sea bass fed diets including defatted BSF prepupae meal (Wang *et al.*, 2019). Similarly, an increase in liver lipid deposition was evidenced in rainbow trout fed diets containing 25 or 50% full-fat BSF meal with respect to FM (Bruni *et al.*, 2020b; Cardinaletti *et al.*, 2019).

Studies performed on zebrafish suggested that the n-6/n-3 ratio is a key factor in determining hepatic lipid accumulation. The higher the ratio value, the higher was the hepatic lipid accumulation (Vargas *et al.*, 2018; Zarantoniello *et al.*, 2018, 2019, 2020b). Finally, Li *et al.* (2017) evidenced a reduction in liver lipid deposition in the herbivorous species Jian carp fed on defatted BSF-based diets.

With respect to glycogen deposition, to date, only a few studies applying a histological approach are available. PAS was used to discriminate the contribution of glycogen in hepatocytes composition of juvenile clownfish (*Amphiprion ocellaris*) fed partially defatted BSF larvae meal (25, 50 or 75% replacement with respect to FM) and rainbow trout fed full-fat BSF prepupae meal (25 or 50% with respect to FM). However, no significant differences among the experimental groups were detected in terms of glycogen accumulation (Cardinaletti *et al.*, 2019; Vargas-Abúndez *et al.*, 2019).

From this overview it is clear that fish responses are not only species-specific but also dependent on fish life stage and on the characteristics of BSF meal (full-fat or defatted). A traditional and well-established method like histology plays a pivotal role in the analysis of fish gut and is still used as gold standard serving scientific research because of its validity and accuracy. These types of analysis could provide the community with essential insights on the effects of BSF meal on fish.

Fourier transform infrared spectroscopy: hyperspectral imaging analysis and biomolecular composition

Fourier Transform Infrared (FTIR) spectroscopy is a well assessed analytical tool for the analysis of biological samples, such as tissues, cells and biological fluids

(Giorgini *et al.*, 2018b; Notarstefano *et al.*, 2019, 2020). The interaction between the electromagnetic radiation in the mid-infrared spectral range (4,000-800 cm^{-1}) and the samples causes vibrational transitions at the level of chemical bonds, allowing to identify the presence of specific chemical groups (Talari *et al.*, 2017). This potentiality coupled with the optical microscopy allows to perform the hyperspectral imaging analysis of selected areas inside the analysed samples. In addition, this tool lets combine the topographical distribution of the most relevant biomolecules (in terms of lipids, proteins, carbohydrates and nucleic acids) with meaningful information on the biochemical composition and the occurrence of specific biological mechanisms and pathways (Giorgini *et al.*, 2018a; Randazzo *et al.*, 2020a; Zarantoniello *et al.*, 2020a).

FTIR spectroscopy has recently been applied to evaluate the physiological responses of fish species to BSF-based diets, including rainbow trout, Siberian sturgeon, and zebrafish. Most of the studies applying this specific technique have been performed on zebrafish, where administration of diets with increasing full-fat BSF meal levels (0, 25, 50, 75 or 100% with respect to FM; Hi0, Hi25, Hi50, Hi75 and Hi100 fish groups, respectively) was tested during the larval, juvenile and adult phases.

As regards the zebrafish larval phase, due to the small sample size, FTIR analysis was performed on lyophilised samples. Statistically significant higher amounts of overall lipids and SFA were found in Hi50, Hi75 and Hi100 groups with respect to control (Hi0) and Hi25, together with a decrease of the unsaturated FA; conversely, no statistically significant changes were detected in terms of protein composition (Zarantoniello *et al.*, 2020b).

In juvenile and adult zebrafish, cryosections (10 μm thick) of specific target organs (such as liver and ovaries) were analysed. In liver, the hyperspectral imaging analysis provided details on the biochemical composition of this organ which were subsequently coupled to the histological data to provide a more comprehensive overview (Zarantoniello *et al.*, 2019, 2020a). As an example, liver samples of juvenile zebrafish fed diets including increasing BSF meal levels (0, 25, 50, 75 or 100% with respect to FM; Hi0, Hi25, Hi50, Hi75 and Hi100 groups, respectively) showed an increment of total FA together with a decrease of both carbohydrates and phosphates. Specifically, in Hi75 and Hi100 liver samples, higher amounts of total lipids and lower ones of proteins were detected, while the lowest glycogen levels were found in Hi50 and Hi75 groups (Zarantoniello *et al.*, 2020a).

Recently FTIR was used to characterise the macromolecular composition of class IV oocytes of zebrafish adult females fed over a 12 months period on BSF-based diets (Randazzo *et al.*, 2020a). Specifically, while similar amounts of SFA,

MUFA and PUFA were found in control and Hi25 groups, Hi50 showed higher SFA and lower PUFA contents. This study suggested that the substitution of FM with BSF meal up to 25% in zebrafish female diet did not affect reproductive performance, while a higher inclusion (50% with respect to FM) resulted in reproductive impairments, specifically in terms of number of spawned eggs.

Since high dietary SFA and n-6 PUFA intake have often been related to behavioural and cognitive impairments in humans and rodents, FTIR was also applied to better understand the FA composition of zebrafish brain in response to dietary BSF meal inclusion. Although specific behavioural tests such as open-field and photic entrainment tests did not evidence differences among the experimental groups, the FTIR analysis highlighted that increasing dietary full-fat BSF meal inclusion levels caused a drastic decrease of unsaturated FA and carbohydrates in zebrafish brain samples. These results suggest possible compensatory pathways developed by the fish (Zarantoniello *et al.*, 2020a).

For commercially relevant species, only a few studies that applied FTIR technique are available. Recently, Giorgini *et al.* (2018a) analysed by FTIR spectroscopy cryosection of medium and hind intestinal tracts of rainbow trout, with emphasis on the mucosa layer. The spectral outcomes were compared to those obtained through the classical histological analysis, based on three different staining methods. The hyperspectral imaging analysis confirmed that the distribution of the most represented macromolecules followed the well-known arrangement of intestine tissues. In addition, the hyperspectral imaging provided a proper outline on the macromolecular composition and the building blocks of rainbow trout intestinal mucosa, through a semi-quantitative information obtained by univariate analysis of the spectral data.

Moreover, the effects of practical diets with increasing full-fat BSF meal levels (0, 25 and 50% with respect to FM) on the macromolecular composition of rainbow trout juvenile's liver have been investigated (Cardinaletti *et al.*, 2019). Results showed that liver samples from fish fed diet with 50% BSF meal inclusion level (with respect to FM) contained higher amounts of lipids and glycogen compared to a control group.

Finally, FTIR was used to assess the liver macromolecular composition and small intestine nutrient absorption in Siberian surgeon fed diets in which 50% of dietary FM was replaced by full-fat BSF prepupae meal (Zarantoniello *et al.*, 2021). As regards liver, a significant decrease of total lipids, FA and glycogen was observed compared to the control group fed a FM control diet. Conversely, no change was observed in the macromolecular composition of small intestine samples.

Based on these results, FTIR spectroscopy represents a reliable tool for the analysis of different fish organs and tissues, providing at the same a correlation between chemical and morphological features and, as further extent, improving histological outcomes.

Microbiome

Dietary composition is one of the key factor in shaping fish gut microbial communities (Egerton *et al.*, 2018; Wang *et al.*, 2018) which in turn can modulate fish metabolism, intestinal mucosa development and maturation, immunity and disease resistance (Llewellyn *et al.*, 2014; Maslowski and MacKay, 2011).

The use of BSF prepupae meal as aquafeed ingredient has been shown to positively affect fish intestinal microbiota biodiversity, regardless of the insects' life-cycle stage and/or defatting process, the insects' dietary percentage of inclusion or the fish species analysed (Huyben *et al.*, 2019; Józefiak *et al.*, 2019a,b; Li *et al.*, 2020a; Rimoldi *et al.*, 2019). Presently, most of the studies on the effects of BSF-based diets on the microbiome have been performed on rainbow trout (Bruni *et al.*, 2018; Huyben *et al.*, 2019; Józefiak *et al.*, 2019a; Rimoldi *et al.*, 2019; Terova *et al.*, 2019).

Bruni *et al.* (2018) explored the effects of 25 or 50% of FM replacement with partially defatted BSF prepupae meal in rainbow trout using the denaturing gradient gel electrophoresis (DGGE), highlighting an increase in intestinal bacterial diversity in fish fed BSF-based diets. DGGE is a well-established, reproducible, rapid and less expensive molecular tool based on genetic fingerprinting. DNA or RNA extraction is followed by the amplification of genes encoding the 16s rRNA and then by the analyses of amplification products by a denaturing gradient gel electrophoresis. In addition, the identification of community members is made possible by the sequencing of excised bands or by hybridisation analyses by specific probes (Muyzer, 1999; Osimani *et al.*, 2019).

An increased biodiversity was reported also by Józefiak *et al.* (2019a), through the fluorescent *in situ* hybridisation, including 20% of full-fat BSF prepupae in rainbow trout diet. This hybridisation technique based on nucleic acids allows the identification of microbial species using group or species-specific fluorescent labelled oligoprobes avoiding the use of DNA extraction and polymerase chain reaction (PCR) (Kumar *et al.*, 2018).

These results were in line with further studies performed on rainbow trout (Huyben *et al.*, 2019; Rimoldi *et al.*, 2019; Terova *et al.*, 2019) that evidenced an increased microbiome biodiversity with dietary administration of up to 30% of defatted BSF prepupae meal, using high throughput sequencing method (Illumina MiSeq). This

next-generation sequencing tool allows to inexpensively produce large volumes of sequence data and to have a higher power of resolution in detecting microbial species compared to conventional methods (McAdam *et al.*, 2014; Metzker, 2010). Illumina sequencing was also utilised in studies performed on Atlantic salmon (Li *et al.*, 2020a) and Siberian sturgeon (Zarantonello *et al.*, 2021) that, in agreement with the previous studies, highlighted an increase in microbial community diversity in fish fed diets including 15% of partially defatted BSF larvae meal or 50% of full-fat BSF prepupae meal, respectively.

A higher microbiome diversity is generally considered as an indicator of improved gut health, while a reduced diversity has frequently been associated to gastrointestinal tract colonisation by pathogens (Apper *et al.*, 2016; Sekirov *et al.*, 2010).

The overall increased diversity and the positive modulation of intestinal microbial communities in response to different levels of dietary BSF meal inclusions have been mainly attributed to chitin. Chitin is generally considered not easily digestible by fish but represents one of the main growth substrates of lactic acid bacteria (LAB) (Askarian *et al.*, 2012; Ringø *et al.*, 2012). LAB are related to *Firmicutes* and *Actinobacteria* phyla which usually represent the 'core gut microbiota' in different marine and freshwater species (Ghanbari *et al.*, 2015; Givens *et al.*, 2015; Li *et al.*, 2014; Ringø *et al.*, 2016; Wong *et al.*, 2013). These bacteria, using chitin as prebiotic, are crucial in making available indigestible carbohydrates leading to a better nutrient accessibility and utilisation for fish (Beier and Bertilsson, 2013). In addition, LAB contribute to the synthesis of vitamins and short-chain FA, considered as the primary enterocyte's energy source (Ghanbari *et al.*, 2015), or of important anti-inflammatory molecules, like butyrate (Rimoldi *et al.*, 2016; Terova *et al.*, 2016).

Firmicutes and *Actinobacteria* phyla have been shown to increase in intestinal mucosa and digesta of rainbow trout fed with defatted BSF prepupae meal (up to 50% of inclusion; Bruni *et al.*, 2018; Rimoldi *et al.*, 2019; Terova *et al.*, 2019) or full-fat BSF prepupae and larvae meal (30% of inclusion; Huyben *et al.*, 2019). In particular, Terova *et al.* (2019) reported that the dietary defatted BSF inclusion increased the relative abundance of *Lactobacillales*, mainly represented by *Lactobacillaceae* and *Leuconostocaceae*, involved in starch and fibres digestion as well as of bacteria from the order *Clostridiales* that includes many butyrate producers like *Clostridium butyricum*.

Similarly, an enhanced colonisation of *Lactobacillus* sp. was observed in rainbow trout fed a diet including 20% of full-fat BSF prepupae meal (Józefiak *et al.*, 2019a) and in Siberian sturgeon fed 15% of full-fat BSF larvae meal (Józefiak *et al.*, 2019b).

As reported by Huyben *et al.* (2019), the abundance of *Bacillaceae* (phylum *Firmicutes*) was higher in rainbow trout fed BSF-based diets and this increase was attributed to the high level of dietary chitin since these bacteria are able to use chitin through the endogenous production of chitinase. Furthermore, the same authors demonstrated that the presence of *Corynebacterium* (phylum *Actinobacteria*) was higher in rainbow trout fed the full-fat BSF larvae or prepupae meal with respect to the defatted larvae meal. This underlies the ability of *Corynebacterium* to use dietary lipid through the activation of endogenous lipases (Brennan *et al.*, 2002). An increased abundance of *Corynebacterium* was also observed in Atlantic salmon fed on partially defatted BSF-based diet (15% of inclusion) with respect to the control diet (Li *et al.*, 2020a).

Furthermore, chitin and its deacetylate derivate chitosan have antimicrobial properties and bacteriostatic effects, particularly on some Gram-negative pathogen bacteria (Nawaz *et al.*, 2018; Qin *et al.*, 2014; Udayangani *et al.*, 2017). This defence mechanism could be enhanced by LAB which are able to produce bactericidal compounds like lactic acid, hydrogen peroxide and biosurfactants preventing the adhesion to intestinal mucosa of pathogens like *Staphylococcus aureus*, *Streptococcus agalactiae* and *Pseudomonas aeruginosa* (Gajardo *et al.*, 2017; Gudiña *et al.*, 2015).

Several studies on rainbow trout fed defatted BSF prepupae meal diets (up to 50% of inclusion; Bruni *et al.*, 2018; Rimoldi *et al.*, 2019; Terova *et al.*, 2019) or full-fat BSF prepupae and larvae meal diets (30% of inclusion; Huyben *et al.*, 2019) revealed a reduction in both gut digesta and mucosal-adhered *Proteobacteria*, a Gram-negative phylum containing pathogens. In particular, Rimoldi *et al.* (2019), reported that 20 or 30% of defatted BSF prepupae meal dietary inclusion increased the number of bacteria belonging to *Mycoplasma* genus which is associated to a beneficial effect on host health due to the production of lactic and acetic acids as main metabolites (Razin, 2006). Similarly, rainbow trout fed 25 or 50% of defatted BSF prepupae meal inclusion in diets were particularly rich in *Pseudomonas stutzeri* (Bruni *et al.*, 2018) which possess antiviral activity and was listed as a probiotic bacterium (Balcázar *et al.*, 2006; Nayak, 2010). In contrast, in rainbow trout, a 20% full-fat BSF prepupae meal inclusion caused an increase in the *Clostridium coccoides* abundance (Józefiak *et al.*, 2019a) which is known to play an important role in fish immune response and pathological processes (Liu *et al.*, 2016).

Finally, recent studies performed on zebrafish fed diets with increasing inclusion levels of full-fat BSF prepupae (0, 25, 50, 75 or 100%) showed contradictory results. In zebrafish larvae, the positive effects of dietary BSF inclusion was highlighted by the highest microbial community

biodiversity showed by the group fed 50% substitution of FM with full-fat BSF prepupae meal as well as by the *Vibrio* abundance which was negatively influenced by the dietary increasing inclusion of full-fat prepupae meal (Zarantonello *et al.*, 2020b). Conversely, the same BSF dietary inclusion levels caused in zebrafish juveniles, a reduction of microbial diversity (Zarantonello *et al.*, 2020a). However, the overall fish gut health was preserved since BSF prepupae meal is particularly rich in lauric acid which is known to possess anti-inflammatory and antimicrobial properties against Gram-positive bacteria (Skřivanová *et al.*, 2005, 2007; Spranghers *et al.*, 2018).

It should be pointed out that to fully unveil the response of intestinal microbiota to BSF dietary inclusion levels, differences between mucosa-adhered and gut digesta communities must be considered since some allochthonous species could poorly colonise host intestine with a lower biodiversity with respect to the intestinal content (Kim *et al.*, 2007). Different studies on BSF prepupae meal administration in salmonids reported that microbial diversity was higher in gut digesta than in intestinal mucosa (Bruni *et al.*, 2018; Li *et al.*, 2020a). For that reason, analysing only gut digesta- or intestinal mucosa- associated microbiota or a mixture of both of them could partially underestimate microbial community response to dietary changes, while it is recommended to profile mucosal and digesta communities separately when feasible.

Molecular biology (real-time PCR): growth factors, immunity, chitinases, lipid metabolism, appetite stimulus

One of the most robust and widely used methods for gene expression quantification is the real-time PCR which is able to distinguish and amplify a specific nucleic acid sequence in a sample, monitoring the amplification process through the correlation between product concentration and fluorescent intensity (Olivotto *et al.*, 2011; Valasek and Repa, 2005; Wong and Medrano, 2005).

This technique has been used in some of the most important finfish species like gilthead sea bream (Psafakis *et al.*, 2020), European sea bass (Kokou *et al.*, 2019), Atlantic salmon (Belghit *et al.*, 2019b), Siberian sturgeon (Luo *et al.*, 2019), sole (Piccinetti *et al.*, 2015), ballan wrasse (Piccinetti *et al.*, 2017) and rainbow trout (Lindholm-Lehto *et al.*, 2019). However, because of the lack of complete and easy genomic information availability about these last species its application is still limited (Ribas and Piferrer, 2014).

With the use of BSF meal in aquafeed formulation, studies about the application of real-time PCR over the last few years are quite limited and mainly focused on growth factors, fish immune and stress response, appetite stimulus, lipid metabolism and chitinolytic activity.

Considering growth factors [insulin-like growth factors (*igf1*, *igf2a*) and myostatin (*mstn*)], BSF meal dietary inclusions usually resulted in good growth performance in most of the studies. In both zebrafish larvae and in rainbow trout juveniles, an increase in growth parameters was well supported by the hepatic gene expression of the growth factors (Cardinaletti *et al.*, 2019; Zarantoniello *et al.*, 2020b). These results support the hypothesis that BSF meal possesses a proper amount of proteins (up to 60% dry weight) and a well-balanced amino acid (AA) profile (similar to that of FM) which are key dietary features to guarantee a proper aquaculture production (Barroso *et al.*, 2014).

Fish growth is strictly related to feed intake and the brain (particularly the hypothalamus) is a key-actor in regulation of energy metabolism, nutrient absorption, and the control of feeding activity. However, it should be considered that the gastrointestinal tract is connected to the brain in metabolic and appetite control. The gut-brain crosstalk occurs through the release of a number of gut peptides that exert responses within the brain, as well as through neuroendocrine and sensory inputs from the gut (Sadoul and Vijayan, 2016). Other signals are also involved such as nutrient levels, through central nutrient sensing systems, and the presence/absence of food in the gastrointestinal tract, through vagal afferents projecting to the brain (Bertucci *et al.*, 2019). To date, studying the gene expression of both orexigenic [ghrelin (*ghrl*), neuropeptide γ (*npv*) and cannabinoid receptor 1 (*cnr1* or *cb1*)] and anorexigenic [leptin (*lepa*) and melanocortin 4 receptor (*mc4r*)] signals represents a valid tool to better understand fish responses to new formulated diets. No significant variation in *ghrl* gene expression in medium and distal intestine of rainbow trout juveniles fed with practical diets containing 30 or 60% substitution levels of vegetable proteins with BSF prepupae meal was recently evidenced (Randazzo *et al.*, 2020b). However, in the same experimental group, but considering brain, appetite signals analysed (*cb1*, *npv* and *mc4r*) showed a significant downregulation (Randazzo *et al.*, 2020b). Differently, in zebrafish larvae and juveniles, gene expression of orexigenic signals (*ghrl*, *npv* and *cb1* analysed in whole fish samples for zebrafish larvae; *ghrl* and *cb1* in intestine samples for zebrafish juveniles) increased with the increasing dietary BSF prepupae meal inclusion levels (Zarantoniello *et al.*, 2020a,b) fully supporting growth.

While BSF meal has been proven to be an adequate protein source, its inclusion in aquafeeds is still possibly limited by the chitin content (Olsen *et al.*, 2006; Spranghers *et al.*, 2017) and its (often) unbalanced FA profile (Ewald *et al.*, 2020).

Over the last years a controversial role has been attributed to chitin in aquafeed formulation (Henry *et al.*, 2015). High dietary inclusion levels of BSF meal (and possibly of chitin) often induced a fish growth reduction (Caimi

et al., 2020b; Zarantoniello *et al.*, 2019, 2021); however, no direct correlation between chitin feed content and fish growth reduction has yet been demonstrated. Chitin can also have a beneficial activity on the fish immune system, lower stress response and improved gut health (Józefiak *et al.*, 2019a; Nogales-Mérida *et al.*, 2018; Ringø *et al.*, 2012).

The use of molecular markers represents a valid and up to date tool since several biomarkers are available, for different fish species, to assess stress and immune response. Studies demonstrated the correlation between increased gene expression levels of glucocorticoid receptor and heat shock proteins and exposure to stressors (including malnutrition; Piccinetti *et al.*, 2015). Pro-inflammatory cytokines such as interleukin 1-beta (*il1b*), tumour necrosis factor α (*tnfa*), anti-inflammatory cytokines like interleukin-10 (*il10*), and inflammation mediators such as nuclear factor kappa-light-chain-enhancer of activated B cells (*nfkbb*) and myeloid differentiation primary response 88 (*myd88*) represent useful markers of inflammation, able to provide early information when testing new ingredients in aquafeed formulation (Fehrmann-Cartes *et al.*, 2019; Marjara *et al.*, 2012; Seierstad *et al.*, 2009). However, the presently available results are still controversial and fish responses seem to be species and stage specific as well as related to BSF meal dietary inclusion levels.

Gut gene expression of immune response markers in adult zebrafish (*il1b*, *il6* and *tnfa*; Zarantoniello *et al.*, 2019), pre-smolt (*il4*, *tgfb1*, *il10*, *ifny*, *il8* and *myd88*; Li *et al.*, 2019) and seawater-phase (*il1b*, *il17a*, *myd88*, *il8*, *il4*, *mhcl*, *il10*, *ifny*, *tgfb1*, *cd8b*, *cd3g* and *foxp3*; Li *et al.*, 2020b) Atlantic salmon was not negatively affected by the BSF-meal dietary inclusion level up to 50, 60 or 100%, respectively.

Conversely, a dietary full-fat BSF inclusion of 50% or higher resulted in the immune response activation in both larval and juvenile zebrafish intestine (*il1b*, *il10*, *il6* and *tnfa*; Zarantoniello *et al.*, 2018, 2020ab) and in rainbow trout juveniles medium intestine (*il10*, *tnfa* and *tlr-5*; Cardinaletti *et al.*, 2019) as well as in the stimulation of regulatory T cell activity in the proximal and distal intestine of Atlantic salmon (*cd3g* and *foxp3*; Li *et al.*, 2019).

Inflammatory events detected by real-time PCR can be coupled with a higher stress response: an increase in the hepatic *hsp70.1* gene expression was detected in rainbow trout juveniles (Cardinaletti *et al.*, 2019) and pre-smolt Atlantic salmon (Li *et al.*, 2019). Accordingly, in zebrafish larvae and juveniles, an increased dietary full-fat BSF prepupae meal inclusion resulted in a higher hepatic *hsp70.1* and glucocorticoid receptor (*nr3c1*) gene expression (Zarantoniello *et al.*, 2020a,b). However, in Atlantic salmon fed diets with higher full-fat BSF meal inclusion (85% of the diet protein content) the hepatic expression of *hsp70.1* was not affected by the diet (Belghit *et al.*, 2019b).

Finally, due to the unbalanced fatty acid profile of BSF meal, with emphasis on their lack of PUFA, a number of biomarkers related to long-chain PUFA biosynthesis and FA metabolism is now available and represents a great opportunity for better understanding the biosynthetic pathways in different fish species in response to dietary treatments. Most of the studies so far published focused their attention on elongase and desaturase gene expression. Specifically, Bruni *et al.* (2020b) demonstrated that the *elovl2* and *fads2* gene expression in the pyloric caeca of rainbow trout juveniles tended to increase with the increasing amount of the dietary full-fat BSF meal inclusion (25 and 50%). Accordingly, in both zebrafish larvae (Zarantoniello *et al.*, 2020b) and adults (Randazzo *et al.*, 2020a; Zarantoniello *et al.*, 2019), an upregulation of *elovl2*, *elovl5* and *fads* hepatic gene expression was observed in experimental groups fed the highest full-fat dietary BSF prepupae meal inclusion (100 and 50%, respectively). The dietary lack of PUFA in freshwater fish fed BSF-based diets could enhance the conversion of shorter-chain FA in highly unsaturated ones through the activation of elongation and desaturation pathways (Tocher, 2010). Furthermore, an increasing expression of markers involved in FA (*cd36*, *fabp2*) and cholesterol (*npc11l1*) uptake has been observed in the proximal intestine of Atlantic salmon fed diets including 60% of full-fat BSF meal (Li *et al.*, 2019).

While real time PCR may represent a valid tool for the early detection of many physiological changes in fish, if applied to nutritional studies its employment should be coupled with other laboratory techniques, like histology and infrared spectroscopy, to obtain a clearer overview. Further research is necessary to overcome the lack of complete and easily available genomic sequences for farmed fish species and to make real-time PCR a 'routine technique' in nutritional studies.

3. Fish quality

The consumers' perception process of food quality has been summarised by Fernqvist and Ekelund (2014). The authors showed how it is influenced by intrinsic quality attributes (i.e. sensory properties) and intrinsic and extrinsic quality cues which compose quality expectations. Among the firsts, attributes perceived before the consumption like colour, size, and damages are listed. The latter aspects refer to label, packaging, and other external factors. In addition to this, consumers are increasingly focusing on health, eco-friendliness, sustainability of food (Fernqvist and Ekelund, 2014) suggesting that people are looking for deeper sensory pleasures from foods, moving from the sensory properties to health and ethical motivations (Lee and Hwang, 2016). On this regard, the aspects related to animal farming, such as animal right, welfare, and feed assurance, are embedded into the 'new' concept of meat quality (Bernués *et al.*, 2003).

To date, researchers have useful tools to objectify many of the quality parameters, such as physical properties, chemical composition, sensory properties, and consumers' liking which have been applied to answer if BSF meal inclusion in fish diets may affect fish quality and consumers' acceptance.

Physical attributes, such as fillet colour, water holding capacity (WHC) and texture are commonly evaluated by instrumental methods. For instance, colorimeters have been utilised by different authors while performing a colour evaluation of fillets from Atlantic salmon (Bruni *et al.*, 2020a) and rainbow trout (Bruni *et al.*, 2020b; Renna *et al.*, 2017; Secci *et al.*, 2019) fed diets with different inclusion levels of partially or not defatted BSF larvae or prepupae meals. This instrument is designed to assess the colour of a sample in a user-friendly way (easy to use and fast response); however, the punctual reading of the colorimeter requires at least two- or three-points determinations in the same sample. Mancini *et al.* (2018) instead, captured the whole fillet image through a digital camera, then the colorimetric measurement was carried out by ImageJ software. Irrespective to the instrument utilised, results are generally presented using the CIELab colour space (CIE, 2018), thus splitting the colour into three axes explaining the lightness (L^*), redness (a^*), and yellowness (b^*) values of the sample. In this way, colour perception and its variations are difficult to interpret. Indeed, data showed as L^* , a^* , and b^* values do not allow an inexpert reader to understand the overall sample colour perception. As a consequence, the differences observed through these methods among the samples did not answer the question: 'were the differences among colour of the samples perceived by an observer?'. A useful tool to distinguish different colours is the Delta E (ΔE) which is defined as a difference in sensation, as revealed by the name itself: Delta is a Greek letter that stands for the incremental change of a variable, while the 'E' is the abbreviation for 'Empfindung', the German word for sensation. As a whole, ΔE shows the distance between two colours and it is calculated as proposed by Sharma and Bala (2002). Furthermore, Mokrzycki and Tatol (2011) reported different ΔE ranges based on the observer perception of the colour difference, as follows: $0 < \Delta E < 1$ – observer does not notice the difference; $1 < \Delta E < 2$ – only experienced observer can notice the difference; $2 < \Delta E < 3.5$ – inexperienced observer also notices the difference; $3.5 < \Delta E < 5$ – clear difference in colour is noticed; $\Delta E > 5$ – observer notices two different colours. While looking at the results showed in the literature, it seems that the dietary treatments with BSF did not affect colour values of the rainbow trout fillets (Renna *et al.*, 2017), with the exception of yellowness which was, in some cases lowered (Bruni *et al.*, 2020b; Mancini *et al.*, 2018). In the case of Mancini *et al.* (2018), where ΔE was proposed, control (C) and fillet from trout fed BSF at 25% of substitution (HI25) showed the same perceived colour (ΔE value=0.39), whereas the ΔE values calculated between fillets from C and trout fed BSF

at 50% of substitution (HI50) or between HI25 and HI50 fillets were 2.96 and 2.60, respectively, thus being perceived as different. The inconsistent results found until now can be due to the complex interactions composing the overall sample colour. For instance, the flesh colour may be directly affected by the pigments contained in the administered diets (i.e. carotenoids), especially carried by vegetable ingredients (soybean, corn) and BSF meal, in a minor extent (Secci *et al.*, 2019). The addition of astaxanthin in the diet for salmonids, inferable from the high a^* values reported for Atlantic salmon (Bruni *et al.*, 2020a) and rainbow trout (Mancini *et al.*, 2018) fillets, could drown out the effect of dietary ingredients on the different indexes. In addition, lipid oxidation might produce yellow pigments, hence high b^* values can be found in fillets with high malondialdehyde equivalents content (MDA-eq.), as in the case of rainbow trout fillets fed on BSF meal-free diet (b^* : 5.57; MDA-eq.: 0.55 mg/kg fillet) or containing 25% of full-fat BSF prepupae meal (b^* : 4.21; MDA-eq.: 0.30 mg/kg fillet) (Bruni *et al.*, 2020b).

Considering WHC and texture, these two physical properties are strictly connected, since the WHC (defined as the ability of a food to retain its own water after the application of a pressure, centrifugation, or heating) has proved to play a key role in the formation of food texture (Picard *et al.*, 2017). The most common method adopted for WHC assessment is the one proposed by Hultmann and Rustad (2002) and modified by Iaconisi *et al.* (2017) based on a centrifugation at $510\times g$ for 5 min. of a 2 g sample with known water content. Applying this method to fillets from BSF meal fed fish, Bruni *et al.* (2020a,b) did not find any significant difference nor among rainbow trout or Atlantic salmon samples. Similarly, Secci *et al.* (2019) did not show any significant WHC modifications of rainbow trout fillets as affected by BSF meal inclusion in fish diet, either after frozen storage (120 days at -10°C) and cooking (boiling at $95-98^\circ\text{C}$ for 5 min.). An indirect measurement of WHC is obtained by calculating the cooking loss as the weight difference of the fillet before and after the cooking process, according to the formula: $100 \times [(\text{raw fillet weight} - \text{cooked fillet weight}) / \text{raw fillet weight}]$. Consistently with the WHC assessment, both Borgogno *et al.* (2017) and Secci *et al.* (2019) did not underline significant dietary effects on this parameter. Texturometer is generally utilised for texture measurements. The instrument can be equipped with a variety of load cells (as 1 kN or less) and probe (cylindrical, straight blade, Warner-Bratzler) based on the type of test the users want (shear test, texture profile analysis, etc.). Other parameters, such as cross-head speed and the percentage of total deformation have to be set. For this reason, results obtained with different methods and by different researchers are not comparable. Despite this, the textural properties of the fillets from fish fed diets containing BSF meal as alternative protein source seemed not to vary with respect to a control diet, irrespective to the

substitution levels (commonly 25, 50, 75% of FM replaced) and the investigated species (rainbow trout: Borgogno *et al.*, 2017; Secci *et al.*, 2019; Atlantic salmon: Bruni *et al.*, 2020a). Analogous outputs were obtained while using *Tenebrio molitor* larvae meal as protein source at 25 or 50% of inclusion for feeding blackspot sea bream, *Pagellus bogaraveo* (Iaconisi *et al.*, 2017) or rainbow trout (Iaconisi *et al.*, 2018). In conclusion, from the instrumentally evaluation of the physical properties of fish fillets emerged that BSF did not impair these items even if colour modification needs to be considered when formulating the experimental diets.

Fish are an important source of energy, essential and non-essential AA, minerals (iodine, selenium, calcium) and vitamins (A, D) and a functional food, being one of the main sources of the long-chain PUFA, as eicosapentaenoic and docosahexaenoic acids (EPA and DHA, respectively). A weekly consumption of around 1.75 g (for adults) and 3.5 g (during pregnancy and lactation) of EPA+DHA is highly recommended while following a healthy diet (EFSA, 2015), since they play positive roles in the prevention of body overweight and obesity (Buckley and Howe, 2009) and in the protection against cardiovascular diseases (Bernstein *et al.*, 2012). Hence, fish quality needs to be evaluated considering flesh chemical composition, by means at least of the AA and FA profiles. The methods utilised for these analyses were well established and no significant implementations have occurred in the recent published articles (Belghit *et al.*, 2018; Borgogno *et al.*, 2017; Bruni *et al.*, 2020b,a; Stadlander *et al.*, 2017). Specifically, total AA composition is commonly evaluated using HCl to hydrolyse fish samples prior to assess a liquid chromatography, as ultra-performance or high pressure ones. Results are commonly expressed as mg AA/g muscle. Concerning FA, once total lipids are extracted from the flesh (methanol:chloroform 2:1 v/v being the most utilised solution) and quantified (gravimetrically), they are subdued to a saponification, then they are methylated to obtain fatty acid methyl esters (FAME). An internal standard as C19:0 (Belghit *et al.*, 2018, 2019a) or C23:0 (Borgogno *et al.*, 2017; Bruni *et al.*, 2020a,b; Secci *et al.*, 2019) is added to the samples. Finally, FA composition is determined using a gas-chromatograph (GC) coupled with a flame ionisation detector. The comparison between the recorded chromatograms with the one of a pure standard FAME mix allows the researchers to qualitatively determine the FA profile, thus the results are expressed as % of total FAME. A quantitative analysis is also possible when using a calibration curve obtained with the FAME standard mix and it is highly suggested. Indeed, the FA content (g/100 g muscle) is necessary to retrieve the fundamental information on the EPA+DHA level in the sample, hence, to know if the samples make a valuable contribution to a balanced and healthy diet. Nevertheless, the FA content of fillet from fish fed BSF meal is still scarcely depicted (Belghit *et al.*, 2019a; Bruni *et al.*, 2020b; Renna *et al.*, 2017; Secci *et al.*, 2019). However, as showed by several studies,

fillet FA profile represents the main weakness of BSF meal inclusion in fish diet, while the total AA both of Atlantic salmon muscle (Belghit *et al.*, 2018, 2019a) and rainbow trout plasma (Sealey *et al.*, 2011) were scarcely affected. Since the early work of St-Hilaire *et al.* (2007), it was evident that the partial substitution of fishmeal (or of the overall protein content) with BSF meal dramatically increased rainbow trout fillet SFA while decreasing the PUFA fraction. Numerous subsequent studies have confirmed this finding irrespective the fish species investigated (Belghit *et al.*, 2018, 2019a; Borgogno *et al.*, 2017; Bruni *et al.*, 2020a,b; Mancini *et al.*, 2018; Renna *et al.*, 2017; Sealey *et al.*, 2011; Secci *et al.*, 2019; Stadlander *et al.*, 2017; Zarantoniello *et al.*, 2019). Since BSF meal is rich in lauric acid (12:0), fillet increase in SFA was expected in fish fed BSF diet. In addition, while adding BSF meal to the diet, a reduction of FO, which is the main source of n-3 PUFA, could be necessary to obtain isolipidic and isoenergetic diets. The sum of these elements leads PUFA depletion in fish flesh which, in turn, lowers the overall nutritional quality of the fillet. For this reason, mitigation actions were investigated such as the use of partially defatted BSF meal (Renna *et al.*, 2017), the use of BSF grown on substrates rich in n-3 PUFA (St-Hilaire *et al.*, 2007; Truzzi *et al.*, 2020), or the avoidance of FO reduction in feeds (Bruni *et al.*, 2020a). However, interesting information comes from the recent research of Bruni *et al.* (2020b) who observed attenuated FA profile differences in rainbow trout fed full-fat BSF prepupae meal (inclusion levels set at 0, 25 or 50%) relative to the dietary differences. Surprisingly, the n-3 PUFA as well as EPA and DHA amounts were not impaired by the dietary intervention, thus resulting in a well-balanced FA profile of the fillets. Standing on our knowledge, Bruni *et al.* (2020b) linked for the first-time fillet quality and fish lipid metabolism, finding that the formulated practical diets containing full fat BSF meal were effective in increasing pyloric caeca *elovl2* and *fads2* gene expression which in turn positively modified the fillet dietary FA profile. This topic deserves further investigations.

The modifications occurring both on lipid and protein fractions while handling fish fillets, i.e. storage and cooking, promote the development of the fish volatile profile (referred as volatile organic components; VOCs). Recently, Nieva-Echevarría *et al.* (2018) found significant different volatiles comparing wild and farmed European sea bass which the authors associated to contaminants and diets. In addition, an interaction between fish growing conditions and cooking methods was highlighted by the same authors, because of a different lipid content and FA profile. Hence, a possible effect of BSF meal inclusion in the diet on farmed fish can be also hypothesised. The most common method to evaluate VOCs composition is based on a solid-phase extraction, followed by the GC-mass spectrometry (MS) analysis of the sample headspace, as described in Iglesias and Medina (2008) and modified by Fratini *et al.* (2012).

For this purpose, the extracted samples were inserted in a vial and heated (60 °C for 30 min.) to collect VOCs in the headspace of the vial. The VOCs were absorbed on a fibre, as Carboxen™/Polydimethylsiloxane (75 µm) (Mancini *et al.*, 2018) prior to be GC-analysed. The identification of components is generally conducted by consulting available libraries and comparing with mass spectra and retention times of commercial standards. At the best of authors' knowledge, the articles showing the VOCs content are limited in literature. Among these, the paper by Mancini *et al.* (2018) offers interesting results while assessing VOCs profile of BSF larvae meal, rainbow trout feeds (control, 25 or 50% of FM substitution with partially defatted BSF larvae meal), and fish fillets. Although the dietary VOCs profile was effectively different, Mancini *et al.* (2018) showed that the VOCs profile of fillets was scarcely affected by the presence of BSF in the diet for rainbow trout, irrespective the substitution level. Such result could be attributable both to a molecule degradation, occurring during the digestive process of the fish, and/or to an absent muscle deposition. An innovative approach was adopted by Bruni *et al.* (2020a) while evaluating the volatile composition of raw Atlantic salmon fed diets containing 25 to 75% BSF meal larvae. The proton transfer reaction-time of flight-mass spectrometer (PTR-ToF-MS) technique offers technical advantages as a rapid data collection (100 s for each sample) and the possibility to evaluate VOCs of the whole sample at room temperature, hence avoiding sample manipulation (mincing, solid phase extraction), and heating. Despite its pros, PTR-ToF-MS technique requires numerous and time-consuming offline activities, corresponding to the spectral-calibration, raw data acquisition and correction (elimination of peaks imputable to water chemistry, interfering ions, and also elimination of all peaks for which the average concentrations are lower than an established threshold), and tentative peak identification based on literature or libraries. This last step appears to be critical, since 18 out of 29 VOCs were quantified without being identified, as depicted in Bruni *et al.* (2020a) results. Concerning the effect of BSF meal on Atlantic salmon, data agree with Mancini *et al.* (2018) since no different VOCs profiles emerged among the samples.

In conclusion, the inclusion of BSF meal up to 25% did not jeopardise fillet physical and nutritional characteristics, while upper inclusion levels mainly affect colour and FA composition. Despite this, understanding if and how the diet affects fillet sensory properties and consumers' liking is necessary to positively judge BSF meal as protein source for aquafeeds. The early study by Sealey *et al.* (2011) approached the topic through a sensory evaluation of rainbow trout fillets conducted by 30 untrained panellists (14 males and 16 females, from 18 to 65 years old). The method adopted was a triangle difference test, which consists to indicate the odd sample in a set of three blinded samples. Panellists were also free to express the reason of

their choice and what were the attributes making different the sample perception. Sealey *et al.* (2011) showed that people were unable to discriminate between fillets from rainbow trout fed a control diet (containing anchovy meal) and the experimental diets containing normal and enriched (with n-3 PUFA) BSF prepupae meal. Recently, the intensity of the perceived sensory attributes of fish fed BSF meal was evaluated by using trained (Belghit *et al.*, 2018; Borgogno *et al.*, 2017) or untrained assessors (Stadtlander *et al.*, 2017). For instance, Stadtlander *et al.* (2017) proposed an organoleptic test on steam-cooked fillets from rainbow trout fed a diet where 46% of FM was substituted by BSF meal. Fifteen untrained panellists were asked to rate different sensory characteristics (odour, colour, texture and taste) on a scale between 0 (does not apply) to 9 (applies fully). Similarly, Belghit *et al.* (2019a) tested raw and baked fillets from Atlantic salmon fed diets at increasing substitution level of FM with BSF meal. Ten trained assessors were asked to evaluate the intensity of several sensory attributes (odour, colour, texture) by using a 15-cm non-structured continuous scale. Results from the previous mentioned studies mostly agreed. Indeed, the sole significant variation detected was a difference in fillet colour even if Stadtlander *et al.* (2017) found darker flesh in fillets from trout fed insect meal, while the salmon fed 66% of BSF meal had fillets significantly less coloured than those of the group fed the control diet (without BSF) in Belghit's *et al.* (2018). An innovative approach distinguishes the work of Borgogno *et al.* (2017), who found that BSF larvae meal inclusion at 20 and 40% in rainbow trout diets affected the fillet sensory profile when assessed through a descriptive analysis and a temporal dominance of sensation (TDS) methods by trained panellists. Several outcomes can be retrieved from the proposed methodology, both in terms of sensorial attributes related to aroma, texture, appearance, and tactile sensations and of the sensory perception process during all the chewing. The main criticism of these methods is about the training sessions. Indeed, both descriptive analysis and TDS require three training sessions of about 60 min. each, that means overall 6 sessions dedicated for training more the time necessary for sensory evaluation. Consumers' expectation and willingness to pay were investigated by Ferrer Llagostera *et al.* (2019) while Bruni *et al.* (2020a) focused on consumers' liking. On one hand, discrete choice experiment (DCE) was chosen by Ferrer Llagostera *et al.* (2019) to understand the preferences of Spanish people towards farmed gilthead sea bream fed diets containing insect meal. DCE is basically an on-line questionnaire which proposes different products (in this case gilthead sea bream fed insect meal) at different price levels in several purchase situations. In the case of Ferrer Llagostera *et al.* (2019), the purpose was to identify the consumers' trade-offs in their choice decision. On the other hand, consumers' test was selected by Bruni *et al.* (2020a) to investigate Italian's liking and intention of re-consumption of Atlantic salmon obtained by administering BSF meal to fish as protein

source in aquafeed. The authors served Atlantic salmon blinded samples at 80 consumers, asking them to express their like or dislike (using a 9-points scale) for a series of sensory attributes. Interestingly, Ferrer Llagostera *et al.* (2019) highlighted that people had a good opinion of gilthead sea bream farmed using insects as feed ingredients but they expected these fish to taste 'less'. On the contrary, the hedonic evaluation proposed by Bruni *et al.* (2020a), conducted under no informed condition (consumers did not know what was the dietary treatment), gave important information. Firstly, people perceived as too pale the colour of fillets from groups fed diets with 66 and 100% BSF meal inclusion levels and the flesh resulted slightly firmer than the control group (0% BSF meal), in line with the instrumental analyses (Belghit *et al.*, 2018). Moreover, Bruni *et al.* (2020a) point out that increasing substitution level of BSF meal (0, 33, 66 and 100%) in Atlantic salmon diet did not counteract consumers' overall liking and intention of re-consumption (>70% of positive answers). The results from Spanish and Italian research groups give reason to hope for a complete consumers' acceptance of insect meal as protein source in the aquaculture sector.

4. Conclusions and future perspectives

Several analytical methods and laboratory techniques are presently available to deepen our knowledge about fish welfare and quality in response to diets including BSF meal. While these laboratory techniques have been used in fish and other animal's responses to all kind of alternative ingredients, a number of studies have recently showed their suitability for a better understanding of fish responses to BSF based diets. Results evidenced that certain dietary inclusion levels of BSF meal are able to promote fish health, welfare and quality of the product. However, there is a lack of information about long-term use of these diets. For this reason, scientists should perform further studies over longer periods of time and possibly over the whole fish life cycle. On this regard, emphasis should be given to nutritional programming experiments as well as the possible effect of these new diets on fish reproduction. The laboratory methodological approaches included in this review article may serve as a starting point for this further research.

Considering these last aspects, the authors suggest a constructive crosstalk between research and industry to sustain the development of a high quality and sustainable aquaculture.

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Conflict of interest

The authors declare no conflict of interest.

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