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PhD IN AGRICULTURAL, FOREST AND FOOD SCIENCES

CYCLE: XXXV

ADVANCED INSECT REARING:
A NEW OPPORTUNITY FOR FEED PRODUCTION

Sara BELLEZZA ODDON

Tutor:
Prof. Laura Gasco

Coordinator:
Prof. Domenico Bosco

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// 1. Summary

Insects are considered a promising alternative to the conventional protein sources for feed production. The researches carried out on this topic not only increases the knowledge, but also provides a scientific basis for legislation. With this purpose, the present thesis is a collection of six scientific publications related to both the insect rearing and the use of insect-derived products in animal nutrition.

2. Introduction

In the twenty-first century, the use of insects for food and feeds has emerged as a relevant opportunity, due to the rising cost of protein sources, population growth and environmental pressures (Ocha et al., 2022). The interest of research institutions and companies in insect farming has been growing over time, for both food and feed production. Food and feeds are closely linked to each other, because an increase in animal productions (meat and eggs) is reflected on the production of feeds. Since the 1960s, the global production of livestock has increased substantially, both in number of animals and in productivity (Michalk et al., 2019). However, the demand for animal products is different in developing and developed countries. In the former scenario, the demand is evolving together with the increasing population growth, while in the second one, a more efficient and sustainable production is requested (Michalk et al., 2019). At the same time, the number of feeds should increase in response to the increase in livestock production. Consequently, given the environmental impact - in terms of deforestation and ecosystem deterioration - of certain protein ingredients that are generally used (soybean and fish meals), it will surely become difficult and unsustainable to satisfy meet future needs. Moreover,

the difficulty of making up for the increase in feed-demand will cause an increase in price of the raw feed materials. For all of these reasons, research is currently being conducted to evaluate all the different raw materials that could be used as valid alternatives. At this juncture, insects are considered promising because they are able to transform agro-industrial waste into an animal protein with a high biological value. Black soldier fly (*Hermetia illucens* – BSF) is the most frequently studied and bred of the EU authorized insect species for aquaculture and monogastric feed production. There are many reasons why BSF has stimulated so much interest: (1) its adaptability, (2) its bio-conversion efficiency and, last but not least, (3) the easy management of its life cycle. BSF larvae can feed on a wide range of organic waste. Moreover, the high adaptability of BSF is demonstrated by its survival capacity, even in unfavourable conditions, such as a high density, out-of-range temperatures and low nutritional value of the substrate (Diener et al., 2010). Because of the easy management of the development stages, yellow mealworm *(Tenebrio molitor* – YM) is the second species considered for feed production, but it suffers from some limitations compared to BSF. In particular, YM prefers a dry substrate, and in the case of an excessive density or nutrient deficiencies, cannibalism can occur (Weaver and McFarlane, 1990). Finally, crickets and silkworm are used less frequently in the feed sector, as a result of their restricted feeding regimes and the difficulties of maintaining their cycle also with low densities (Cappellozza et al., 2005; van Huis, 2022). In conclusion, insect products seem to be a promising solution to the present and future socio-enviro-economic problems linked to protein source recruitment. Hence, the aim of the present PhD research thesis is to contribute to the scientific knowledge on this topic. In particular, experimental trials were conducted on both insect rearing and on the use of insect-based products,

mainly BSF, as a feed ingredient and/or an environmental enrichment practice for aquaculture and poultry farming.

3. The protein ingredients of conventional feeds

Soybean meal is the most frequently used protein source for feed production, and this is followed by fishmeal (FAO, 2015). A total of 44% of protein soybean meal is produced by cracking, heating and flaking soybeans, and reducing the oil content through the use of solvents: the extracted flakes are then ground into meal (International Buyers' Guide, 2015). Instead, fishmeal is obtained by means of cooking, pressing, drying and grinding fresh raw fish or shellfish (anchovies, sprat, herring and krill) (EUMOFA, 2021). In some cases, these fish are caught by chance during fishing operations, but in most cases the catch is exclusively destined for fishmeal production (EUMOFA, 2021). However, both of the aforementioned protein ingredients have an economic and environmental cost. From an economic point of view, the market demand for soybean and fish meal is absorbing the supply (EUMOFA, 2021; Ritchie and Roser, 2022). From 1960 until now, the annual soy production has increased by 300 million tonnes. This increase in production is partially derived from improvements in the yields and the extension of land use. In the soy scenario, the expansion of croplands is clearly the main driver of the exponential production that has taken place (Ritchie and Roser, 2022). Considering the same period, fishmeal has undergone a fluctuating production, overall registering an increase of only 7 million tonnes (Pauly et al., 2020). The different production patterns for the two protein sources are the result of their availability. As regard soybean meal, a production increase is still possible, considering natural territories can still be changed into cultivable lands, while marine resources are already scarce. This

aspect is evident if aquaculture production (increased of 104 million tonnes) is compared, again between the 60s and today, with fishmeal production (Pauly et al., 2020). Since marine resources are not rapidly renewable, the fishmeal production trend cannot follow the aquaculture expansion one.

On the basis of the above-reported background and the importance of safeguarding the environment, research is now evaluating new sustainable feed protein sources that have comparable effects on livestock growth performance, product quality and safety with those of conventional sources. Among the protein sources that are available, insects are considered to be one of the most promising.

4. Insect legislation

Since insects are included in the farm-animal category (animals that are kept for the production of food, feeds or other derived products – EU Regulation 1069/2009), the raw materials used as a substrate for their growth must also be subject to the restrictions on productive animals (EU Commission Regulation 2022/1104). Consequently, insects can be fed materials of vegetal origin and some materials of animal origin, such as milk, eggs and their products, honey, as well as rendered fat and blood products from non-ruminants. The use of other slaughterhouse or rendering derived products, manure, or catering waste is prohibited, while the use of unsold products from supermarkets is possible, but only if these do not contain meat or fish.

EU Regulation 2017/893 authorised the use of insect proteins originating from seven insect species – namely black soldier fly (*Hermetia illucens*), common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta

domesticus), banded cricket (*Gryllodes sigillatus*) and field cricket (*Gryllus assimilis*) – in aquaculture feeds. In 2021, silkworm (*Bombyx morii*) was added to the list (EU Regulation 2021/1925) and the possibility of using insect proteins in feeds was extended to poultry and swine (EU Regulation 2021/1372). Figure 1 summarises the possible use of insect-derived products in feed production. As regard live larvae, they can be used in aquaculture, poultry, swine and pet-food, but only if authorised by the competent national authorities of the country where the product is being commercialised (IPIFF, 2022).

Insects as feed - **Regulation (EU) No 68/2013** on the Catalogue of feed materials and in accordance **with Regulation (EC) No 999/2001 and Regulation (EC) No 1069/2009**	Ruminant animals	Aquaculture	Poultry	Pigs	Pets	Fur and other animals (e.g. zoo)	Technical uses(e.g. cosmetic industry, bio-based fuels, production of other bio-based materials such as bioplastics)
Insect proteins (under entry 9.4.1. 'Processed animal protein')	✗	✓ **	✓ **	✓ **	✓	✓	✓
Insect fats (under entry 9.2.1 'animal fat')	✓	✓	✓	✓	✓	✓	✓
Whole insects (untreated) (under entry 9.16.2. 'terrestrial invertebrates, dead')	✗	✗	✗	✗	✓ *	✓ *	✓
Whole insects (treated- e.g. Freeze drying) (under entry 9.16.2.'terrestrial invertebrates, dead')	✗	✗	✗	✗	✓ *	✓ *	✓
Live insects (under entry 9.16.1 'terrestrial invertebrates, live')	✗	✓ *	✓ *	✓ *	✓ *	✓ *	✓
Hydrolysed insect proteins (under entry 9.6.1. 'Hydrolysed animal proteins')	✓	✓	✓	✓	✓	✓	✓

*if authorised by the national competent authority of the country where the product is being commercialised under the specific conditions applicable to processed pet food (in case the product is intended for use as processed pet food)
** Limited to 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*), Field Cricket (*Gryllus assimilis*) and Silkworm (*Bombyx mori*).

Legend	Colour
Restriction to insect species (insect PAPs for aqua feed)- Regulation (EU) **No 142/2011; Annex X Chapter 2 Section 1, A.(2).** - Insect PAPs must be produced in **processing plants approved** in accordance with Article 24(1)(a) of Regulation (EC) No 1069/2009 and **dedicated exclusively** to the production of products derived from farmed insects **'Regulation (EC) No 999/2001; annex IV, chapter IV, Section F, 1 (a)**. - Insect PAPs must be produced according to **processing methods 1 to 5** or **processing method 7 (Regulation (EU) No 142/2011, Annex X, Chapter II, Section 1, B (2).**	(blue)
No restriction as to the insect species (provided that these are not pathogenic to humans and animals)	(yellow)

Figure 1. Authorized use of insect-derived products as feed ingredient. Source: IPIFF – Guide on Good Hygiene Practices (2022)

5. Waste for insect breeding

A wide range of waste and by-products have been tested as BSF growing substrates, such as food waste, agro-industrial waste, manure, abattoir and

municipal organic waste (Siddaqui et al., 2022). The first experimental trials mentioned in literature were aimed at assessing the use of BSF for waste management purposes, and at evaluating the larva survival rate and the growth performance, without considering the nutritional values of the substrates (Newton et al., 2005; Lorimor et al., 2006; Sogbesan et al., 2007I). In such a scenario, the final aim was to determine the feasibility of insect breeding. However, to date, the maximisation of production – while maintaining the concept of a circular economy - is one of the most discussed issues. For this reason, the formulation of the growing substrate has become the basis to efficiently link the production, the local waste utilisation and the economic and environmental impact. Gold et al. (2020) demonstrated that the growth performance of larvae developed on a waste-mixture is improved, compared to those developed on a single type of waste. Moreover, the formulation of a substrate could allow different types of waste with a low nutritional value, which alone would be discarded because unusable, to be used with others with high nutritional value. In addition to the nutritional composition, other aspects should be considered in order to obtain an optimal substrate for BSF larva growth, such as the moisture content, the water holding capacity of the ingredients, and the interaction between the waste and the particle size. Nevertheless, most of the above-listed factors still need to be investigated in depth.

Finally, research studies on the use of non-authorised waste could contribute to the creation of a solid scientific background concerning their insertion into the raw material catalogue for feed production. Specifically, the assessment should not only consider the larva growth performance, but also the microbiological safety of the obtained products. All these aspects could support legislators in extending the list of authorised feed ingredients.

6. The nutritional requirements of insects

Different factors, such as light, humidity, temperature, strain, substrate composition and texture, influence the development of insects, and research is focusing on all these aspects (Chapman, 2013; Bosch et al., 2020). Among the abiotic agents, the determination of the nutritional requirements of insects is one of the least investigated. The selection of an adequate substrate could guarantee not only growth maximisation, but also animal health (Makkar and Ankers, 2014). Some insects, such as BSF, can develop on a wide range of organic material, but – as reported in literature – the larva biomass can change in relation to the used substrate (Singh and Kumari, 2019). As mentioned in the previous paragraph, a reasoned substrate formulation could support the selection, in different proportions, of several kinds of waste, while maintaining a good production performance (Gold et al., 2020). Some nutritional aspects are known and have been generalised for all insects and, considering that they are the largest class in the animal kingdom, makes this knowledge a suitable starting point for specific studies on breeding-interest species. For example, some nutrient requirements can be determined for a single generation, such as amino acids, carbohydrates and lipids, while micronutrients (minerals and vitamins) are only evident after two or more generations (Chapman, 2013). The nutritional needs can be determined, as for any other livestock animal, by using artificial diets, which are also called semi-purified diets, and by establishing the performance of the insects (survival, growth and development time) (Chapman, 2013). Semi-purified diets are composed of natural ingredients (i.e., starch and caseins) in a relatively pure form (nearly 100%) (Lagoc, 1989). Research trials have been carried out to determine the optimal energy requirement and the protein and carbohydrate ratio for BSF (Berragán-Fonseca et al., 2020;

Georgescu et al., 2021). High percentages of carbohydrates and crude proteins (35-55% and 21.9% on a DM basis, respectively) in the diet have been found to increase the development time (Oonincx et al., 2015; Cammark et al., 2017; Barragán-Fonseca et al., 2019). Protein percentages above 32% determine a reduced growth performance and a lower survival rate than 22% (Tschirner and Simon, 2015). On the other hand, since BSF larvae are able to metabolise any carbohydrate excess, through their physiological systems, and convert them into fat reserves, high carbohydrate concentrations (40-60% on DM) do not generate any deleterious consequences on larval development (Barragán-Fonseca et al., 2020; Cohn et al., 2022). The energy content of the substrate is influenced, logically, by the macronutrient levels, but the larva weight tends to increase as the energy value of the feed increases (Georgescu et al., 2021). However, the results obtained so far represent just a small part of all the research that needs to be carried out on insect nutrition. Moreover, although some macronutrient levels have already been evaluated, the specific composition and limiting factors have not yet been identified (i.e., amino acids). Finally, the digestibility of nutrients, which affects the parameters that are evaluated during the formulation of a diet, is a subject that still requires investigation.

7. Insect meal in aquaculture

The scientific literature on the use of insect meal in aquaculture is wide and varied. Naturally, the most frequently tested fish species are carnivorous, that is, both fresh and salt-water fish. Instead, insect meal is mainly derived from BSF and YM. In addition to the species variable (fish and insect), the insect development stage, the type of meal (whole or defatted), the applied treatment method (inactivation and/or

transformation), and the level of insect meal inclusion all affect the growth performance of fish. The use of whole BSF and YM meal, at an inclusion level of 18%, has generated a difference in the performance of rainbow trout (*Oncorhynchus mykiss*). Fish fed with YM in fact performed better, in terms of final body weight (FBW), specific growth rate (SGR) and feed conversion ratio (FCR) than the BSF ones (Melenchòn et al., 2022). Reyes et al. (2020) obtained the same outcomes for seabass with the same inclusion level. On the other hand, Józefiak et al. (2019) did not witness any insect effect (whole BSF and YM meals, 20% inclusion level) on FBW, SGR or FCR. A lower BSF inclusion level (11.9%) decreased the growth performance of largemouth bass *(Micropterus salmoides*), compared to the control diet (Fischer et al., 2022). On the other hand, 20% and 16.8% of full-fat BSF larva meal inclusion in the diets of Atlantic salmon (*Salmo salar*) and Nile tilapia (*Oreochromis niloticus*), respectively, increased the FBW and kept the FCR equal, compared to the control diet (Weththasinghe et al., 2021; Wachira et al., 2021). Therefore, the performance results are closely related to the fish species. Defatted BSF meal seems to determine a better growth performance in rainbow trout than whole meal. In fact, the FCR, feed intake, FBW and SGR of rainbow trout did not differ for BSF and YM dietary treatments, in which the defatted insect meal was included up to 20% (Tran et al., 2022). Although the literature is not exhaustive on the subject, BSF prepupa and pupa meals have also been evaluated. Rainbow trout fed a diet with 30% of BSF prepupa meal displayed lower values of FBW and SGR than the control, but equal FCR levels, while an inclusion of 45% of partially defatted pupa meal did not affect the same parameters (Hoc et al., 2021; Cardinaletti et al., 2022). In short, whole YM meal had no negative effect on the growth performance of the considered aquatic species. On the other

hand, in some cases, whole BSF larva and prepupa meals seemed to reduce the production performance. Hua (2021), who conducted a meta-analysis, described the same scenario, and found that YM seemed to be well tolerated by fish, compared to BSF. Since in the trials of both Józefiak et al. (2019) and Melenchòn et al. (2022) BSF meal was included in similar percentages and in substitution of fishmeal, the observed variable results were probably due to the influence of the breeding substrate and the processing treatments on the chemical composition of the meal. The nutritional and, consequently, the quality variation of insect meal is currently the main limitation to the use of this protein source as a feed ingredient (Liland et al., 2021).
The studies carried out until now represent a good basis to understand the feasibility of insect meal use, which is possible, albeit with some tricks, and to open new research lines about the improvement of its applications in aquaculture.

8. Live larvae as an environmental enrichment practice in poultry

There are several definitions of the "animal welfare" term in literature. Animal welfare should be defined in terms of the natural living, feelings and functioning of animals or as the union of all these aspects (Fraser, 1997). Since the issue of animal welfare implies ethical, economic and political dimensions, it cannot only be linked to the scientific world (Lund *et al*., 2006). In fact, the origin of animal welfare can be traced back to 1964, when the writer Ruth Harrison wrote *Animal Machines* to denounce animal abuse on intensive chicken farms. In response to this document, the British Government appointed a committee to investigate intensive livestock farming and, in the following year, Brambell's report was

published with Five Freedoms in "embryonic form" (McCulloch, 2013). This was a list of different types of behaviour that breeding animals should have the possibility of attaining: standing up, lying down, turning around, grooming and stretching their limbs (Brambell, 1965). The Five Freedoms, listed hereafter, originate from the behaviour described by Brambell and they are still used today for the analysis of animal welfare: (1) Freedom from thirst, hunger and malnutrition, (2) Freedom from discomfort, (3) Freedom from pain, injury and disease, (4) Freedom to express normal behaviour and (5) Freedom from fear and distress. The fourth freedom involves expressing normal behaviour, or rather having the opportunity of exhibiting natural attitudes. Considering poultry, the most common types of natural behaviour are locomotion activities, feather pecking, aggressive behaviour, scratching, dust bathing and nesting (Bhadauria and Bhanja, 2017). Despite the existence of European Directive 2007/43/EC, which guarantees a minimum space for animals in intensive farming (the density coefficient cannot be any higher than 33 kg per m^2), the natural behaviour of poultry is not always manifested nor it is exacerbated and considered "abnormal". For example, feather pecking is one of the most serious behavioural problems in laying hen farming. Apart the negative effect on animal welfare, feather pecking also has an economic impact. Heat loss from defeathered birds can lead to more than 40% higher energy needs or 27% greater consumption (Bilčík and Keeling, 2010). Several theories exist regarding the cause of feather pecking, one of which claims that it evolves from redirected ground pecking (Blokhuis 1986; Bilčík and Keeling, 2010). Aggressive behaviour is also observed on intensive broiler chicken farms. Moreover, in this scenario, the reduced activity of the animals, especially at the end of the growing phase, can be considered a negative aspect of the animals' condition (Ipema et al., 2020).

Modification of the environment of captive animals to increase their behavioural possibilities and, as a consequence, to improve their biological functioning is named environmental enrichment (Newberry, 1995). However, most poultry environmental enrichments are still very limited, because their use is often restricted to a single or a few locations in animal enclosures (Riber et al., 2018). The main enrichment proposals include the provision of perches, nesting areas, and dust bathing substrates (Ferrante, 2008). None of these enrichments pertains to the search for food or therefore stimulates ground pecking behaviour. For this reason, the use of live insect larvae - if properly administered - could be an excellent environmental enrichment practice. Since, in nature, birds feed on insects, the addition or inclusion of live larvae to a commercial diet could guarantee the manifestation of behaviour that is part of the poultry ethogram. A study conducted with older laying hens showed that birds fed live BSF larvae (10% of the daily feed intake) had the same production performance as the control group, but their feather condition underwent an improvement (Star et al., 2020). As far as feed consumption is concerned, the use of 10% BSF live larvae in substitution of a commercial feed was not sufficient to reduce the consumption of the concentrate, while a level equal to 20% determined a reduction of 25% of the ingested feed (Tahamtani et al., 2021). The inclusion of 10% of live BSF larvae in the feeds of broiler chickens generated positive effects on animal activity and leg health, with the best outcome being observed for a frequent administration (4 times per day) (Ipema et al., 2020). However, the only tested insect species in the above reported trials was BSF. Nevertheless, the use of other live insects could probably affect both the production parameters and animal welfare in different ways. Further research is needed to assess the insect preference of birds and whether there are any

differences in the previously discussed parameters as a result of feeding on different insects.

9. Aim of the project and scientific contributions

The first of the two aims of the present PhD project has been to determine a research method for the determination of the nutritional requirement of BSF in order to create a starting point for the formulation of waste-based diets that would be able to maximise the larva production performance. The second goal has been to evaluate insect meal as a feed ingredient in rainbow trout diets and the administration of live larvae as an environmental enrichment in poultry farms.

The present thesis includes a total of six publications:

- 1st aim: two scientific contributions on the determination of the nutritional requirements of BSF;
- 2nd aim: four scientific contributions on the use of insect-derived products in animal feeds:
    - two on the inclusion of BSF defatted meal in rainbow trout diets;
    - two on the use of live BSF larvae as an environmental enrichment on broiler chicken farms.

Moreover, other scientific articles related to the PhD project have been published, and an exhaustive list is reported on page 103.

Isoenergetic-practical and semi-purified diets for protein requirement determination in Hermetia illucens larvae: consequences on life history traits.

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RESEARCH **Open Access**

Isoenergetic-practical and semi-purified diets for protein requirement determination in *Hermetia illucens* larvae: consequences on life history traits

Sara Bellezza Oddon, Ilaria Biasato* and Laura Gasco

Abstract

Background: Black soldier fly (BSF) is one of the most promising species for the intensive breeding of insects given its adaptability and its efficiency in the conversion of waste. To maximize the production and use waste as substrates, it is essential to determine the larvae nutritional requirements. The study aims to evaluate the effects of 5 practical, semi-purified and isoenergetic diets (PSPID) with increasing protein levels (10%, CP10; 14%, CP14; 16%, CP16; 19%, CP19) on BSF life history traits. A total of 2000 six-day-old larvae were weighed and divided into groups of 100 (4 replicates/treatment [PSPID and Gainesville diet (GA) used as environmental control]). In larva-prepupa stage, sampling was carried out on 30 individuals/replicate, while in prepupa-adult stage on 35.

Results: In the whole larval stage, the CP16 treatment performed better when compared to the other groups. At 18 days old, the CP14 treatment showed a higher weight than the CP19 ($P < 0.01$), while the CP10 and CP16 weights were intermediate. On the contrary, the CP10 prepupae weight was higher than the CP19 ($P < 0.01$). The CP10 and CP14 pupae performed better in terms of weight when compared to the others ($P < 0.01$). The dietary treatments did not affect the adult parameters. The sex significantly influenced both the exuvia weight, which was the greatest in females ($P < 0.001$), and the fly lifespan, longest in males ($P < 0.05$). Fly lifespan was also influenced by the interaction between treatment and sex, with the CP19 females showing a longer life than the others ($P < 0.05$).

Conclusions: In conclusion, the optimal protein level considering the whole larval stage is 16% on dry matter (DM). At 18 days old, looking at the size intended for the meal production, it can be considered 14% on DM. The result obtained on adult emergence in PSPID may not be attributable to the protein content. Further researches on macronutrients requirements determination have to be conducted to evaluate the substrates composition effects on BSF life history traits.

Keywords: *Hermetia illucens*, Life history traits, Nutritional requirements, Semi-purified

Background

In recent years, the number of studies and commercial development related to the insect production for recycling, reutilization and reuse of waste biomass from agri-foood system has significantly grown [1, 2]. In Europe, the insect species that can currently be bred for fish, poultry and pig feed production are as follows: the black soldier fly (*Hermetia illucens*), the housefly (*Musca domestica*), the yellow mealworm (*Tenebrio molitor*), the

* Correspondence: ilaria.biasato@unito.it
Department of Agricultural, Forest and Food Sciences, University of Turin, Largo P. Braccini 2, 10095 Grugliasco, TO, Italy

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lesser mealworm (*Alphitobius diaperinus*) and three different species of cricket (*Acheta domesticus, Gryllodes sigillatus, Gryllus assimilis*) (Reg. UE 2021/1372). Among them, the black soldier fly (BSF) is one of the most promising for the intensive breeding, given its adaptability, its efficiency in the conversion of waste, and the simple management of its life cycle compared to the other insect species [3].

According to scientific publications, the black soldier fly larvae can be fed a wide range of organic materials [4] such as restaurant waste [5], vegetable and fruit waste [6], rice straw [7], poultry manure and human faeces [8].

The BSF breeding is focused not only on the re-utilization of waste materials as feed resources, but also on the maximization of the production (which is the primary goal of the livestock farming). However, these two aspects partially contrast with each other. Indeed, the maximization of the production is generally achieved with a standardized process that is difficult to obtain using only waste as substrates, since they are characterized by a strong seasonality and linkage with the territory. In fact, the produced raw materials (food/feed destination) have an environmental vocation and, consequently, the same applies for the resulting waste. However, unlike the raw materials, in order to respect the circular economy model, the displacement of waste is incoherent.

Therefore, the gap between these two aims needs to be reduced in order to create a sustainable insect farm. To achieve this, it is fundamental to determine the nutritional requirements in larval stage and, in turn, formulate diets that maximize the production using waste. The scientific literature concerning the assessment of BSF larvae dietary macro-element requirements is still very limited. In the last few years, researchers have mainly focused on the evaluation of the effects derived from the interaction of macro-elements, for example protein (P) and carbohydrate (C). In particular, Barragan-Fonseca et al. [9] have formulated diets with different P:C ratio (10:35, 10:45, 10:55, 17:35, 17:45, 17:55, 24:35, 24:45, 24:55, respectively) containing chicken feed in a fixed amount and different quantities of starch, casein and cellulose. Furthermore, Cammack and Tomberlin [10] took into consideration not only the P:C ratio (7:35, 21:21 and 35:7, respectively), but also the association with different moisture percentages. Both the studies revealed that diets containing high amount of carbohydrate increased the development time in black soldier fly larvae. Different protein levels in substrates composed by food by-products have also been reported to influence the BSF larval development, with the fastest growth being observed on the high protein and fat diets (CP: 21.9% of the dry matter [DM]; EE: 9.5% DM) [11]. Furthermore, the larval yield and individual pupal weight were the greatest in the diet with the highest carbohydrate content for all the three protein levels (10%, 17% and 24%) [9].

Apart from investigating the relationship between the different macro-elements, it is also necessary to determine the optimal levels of each individual nutrient for the BSF larvae. The currently available information in other animal species could lay the foundations for pilot studies in insects. Indeed, nutritional needs of the animal species used for zootechnical or experimental purposes have usually been determined by using purified or semi-purified diet [12–15]. The purified diet is a feed made out of refined ingredients with precisely defined composition (i.e, starch and casein), while the semi-purified diet contains some natural ingredients in a relatively pure form (nearly 100%) in combination with purified ingredients [16]. Finally, another type of experimental diet to test new ingredients in feed is the practical diet, which is formulated from natural ingredients as cereal grains, oil seed meals and fish meal [16].

Based on the above-reported background, the present study aims to evaluate the effects of different practical, semi-purified and isoenergetic diets (PSPID) with increasing protein levels on larval development and mortality, and adult parameters.

Material and method

Experimental diets

In the trial, five different experimental diets were tested: four practical semi-purified and isoenergetic diets with increasing protein levels (10%, CP10; 14%, CP14; 16%, CP16; 19%, CP19), and the standard Gainesville diet (GA) used as environmental control (Table 1). To maintain experimental diets isoenergetic, a reduction of lipid was performed by decreasing the corn inclusion. The diets were considered practical and semi-purified since they consist of raw materials derived from cereal production (i.e. corn and rice husk) and semi-purified ingredients (casein and starch). The GA was composed by corn, wheat bran and alfalfa as indicated by Hogsette [17]. The GA treatment was considered as the environmental control in order to exclude the possible effects of both the microclimatic alterations and the handling procedures on the obtained results.

As a first activity, the corn was ground to 2 mm diameter by cutter (CL/5 Fimar, Italy) before being mixed with the semi-purified components (casein and starch), and the obtained mixture was kept at 4 °C. Before being used, the mixture was acclimated for 4 h at 28 °C. Just before the actual usage, the whole rice husk and 116.6 g of water (T = 28 °C) to reach the 70% of moisture were added to the meal ingredients (50 g). The whole rice husk (10% of DM) was included in the formulation and added before the substrate use to prevent an excess

Table 1 Ingredients (g/kg as is), chemical composition (g/100 g as is, unless otherwise stated), and gross energy (MJ/kg as is) of the five experimental diets

Items	CP10	CP14	CP16	CP19	GA
Ingredients, g/kg					
Whole rice husk	100	100	100	100	–
Chopped rice husk	237.5	311.4	374.2	410.2	–
Starch	300	230	170	135	–
Casein	62.5	96.6	130.8	164.8	–
Corn	300	262	225	190	200
Alfalfa	–	–	–	–	300
Wheat bran	–	–	–	–	500
Total	1000	1000	1000	1000	1000
Chemical composition[a]					
DM	87.69	89.40	89.74	90.22	88.30
CP as is	9.97	13.95	16.54	19.42	13.55
EE as is	1.27	1.07	0.98	0.92	2.22
Ash as is	5.00	6.40	7.48	8.29	5.79
aNDFom as is	23.77	25.77	33.81	32.05	34.91
NSC[1] as is	49.13	55.78	67.27	68.98	67.00
GE, MJ/kg as is	15.95	16.03	16.20	16.32	16.09

Legend: *CP10* 10% CP diet, *CP14* 14% CP diet, *CP16* 16% CP diet, *CP19* 19% CP diet, *GA* Gainesville diet. *DM* dry matter, *CP* crude protein, *EE* ether extract, *GE* gross energy, *aNDFom* amylase neutral detergent fiber organic matter, *NSC* non-structural carbohydrates.

[a]Values are reported as mean of duplicate analyses

[1]Calculated as 100 - [(100 - DM) + CP + EE + Ash + aNDFom]

substrate cohesiveness. A sample of each substrate was frozen at − 20 °C for the chemical analysis.

Chemical analyses

To properly formulate isoenergetic diets with increasing protein levels, before the diet formulation all the raw materials were chemically analysed. In particular, the dry matter (DM; AOAC #934.01), the crude protein (CP; AOAC #984.13; conversion factor N × 6.25) and the ash (AOAC #942.05) were determined by the International AOAC [18], the ether extract (EE; AOAC #2003.05) by the International AOAC [19] and the amylase neutral detergent fiber organic matter (aNDFom) by Mertens [20]. The NSC was calculated using the following formula:

$$NSC = 100 - [(100 - \mathrm{DM}) + \mathrm{CP} + \mathrm{EE} + \mathrm{Ash} + \mathrm{aNDFom}].$$

The gross energy was analysed using an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany). Moreover, after the substrate preparation, a sample of each dietary treatment was analysed with the same procedures listed above to control the accuracy of the substrate formulation.

Larval stage

The experiment was performed using the BSF colony established at the Experimental Facility of the Department of Agricultural, Forest and Food Sciences (University of Turin). Collected BSF eggs were incubated and the 1-day-old larvae were harvested and fed the GA until the beginning of the trial.

A total of 2000 six-day-old larvae were weighed (pool of 10 larvae per single weight; initial larvae weight: 0.07 g ± 0.02) with a precision balance (Kern & Sohn GmbH; Balingen, Germany; d = 0.001), randomly divided into groups of 100 and allocated in 20 boxes (10 cm × 17.5 cm × 7 cm) covered by a perforated cup (4 replicates per dietary treatment). Each box was filled with 50 g of substrates (0.5 g/larva) that underwent an acclimatization period of 1 h in the climatic chamber in order to avoid a thermal shock for the larvae. The boxes were placed in the climatic chamber with controlled condition (T°: 28 ± 0.5 °C; RH: 70 ± 5%; 16:8 L:D). Every day, the boxes were checked and the substrate was gently mixed with a spoon to prevent the casein surfacing (superficial sedimentation). Throughout the trial, 50 g of feed was added in all the replicates (each treatment received 0.08 g/larva/day) every 4 d until the prepupae appearance (18 days of age). After the beginning of the trial, a total of three samplings (30 larvae/replica) at 4-day intervals were carried out when larvae were 10 (T1), 14 (T2) and 18 (T3) days old to evaluate the growth. Before weight recording, the larvae were cleaned with warm tap water (28 °C) and gently dried with a paper. As the procedure was not destructive, once weighted, the larvae were returned to the box. At the end of the experiment, when 40% of the prepupae were identified, the total number of larvae was counted for the survival calculation.

Prepupal stage

Once the appearance of the first prepupae, no substrate was furtherly added to the boxes. An inspection was made every day to count the prepupae. When 40% of the prepupae was identified, the larval development was considered ended and a sampling was carried out (30 prepupae/replica) to evaluate their weight.

Pupal stage

In comparison with the prepupa, the pupa is characterized by a rigid puparium and the absence of elasticity [21]. After being identified, the pupae weight was recorded and the larva-pupa phase duration (L-P) was determined (35 individuals/replica). A total of 35 pupae per replica were positioned in perforated plastic boxes (10 cm × 8 cm × 8 cm; 1 pupae/box) and placed inside the climatic chamber (T°: 28 ± 0.5 °C; RH: 70 ± 5%; 16:8 L:D). To assess the time for the adult emergence, the pupae check was carried out twice a day.

Adult stage

The days needed for the adult emergence for each pupa (pupa-fly phase duration [P-F]) were recorded. Once fly emerged, the sex of the fly and the weight (fly live weight [FLW]) were recorded. The flies were not fed during their lifetime.

In order to record the FLW, the containment boxes were individually weighted. The FLW was then calculated as the difference between the weight of the box with the fly and the exuvia, and the weight of the empty box and the exuvia recorded at the fly death. The flies were kept in the same climatic chamber of the larvae, prepupae and pupae. At the death, the dead fly weight (DFW) and the exuvia weight (EW) were recorded. The DFW was used for the calculation of the weight reduction (WR) percentage between the FLW and DFW. Moreover, the fly life span (FLS, days) was also evaluated. In absence of fly emergence, after 1 week since the death of the last fly per each experimental treatment (minimum time: 3 weeks, maximum time: 1 month), the pupae were declared dead and the emergence rate (ER) was calculated.

$$ER = \frac{n^{\circ} emerged\ flies \times 100}{n^{\circ} pupae\ collected\ per\ treatment}$$

The effect of the sex on adult stage parameters was finally assessed.

Development score

With the aim of setting up a development score, before the beginning of the trial, a total of 200 pupae in different intra-puparial stage were dissected in order to evaluate their development status. On the basis of this preliminary study (data not shown), in addition to the research carried out by Barros-Cordeiro et al. [22], a photographic-development score has been fine-tuned. A score from 0 to 4 was assigned based on the characteristics of the fly (Fig. 1). The 0 corresponded to the absence of formation, 1 to the anatomical formation of the fly without pigmentation, 2 to the presence of the fly with pigmented eyes, 3 to the fly with the pigmented thorax, and 4 to the presence of an anatomically complete fly. At the end of the trial, all the dead pupae were dissected to determine the development stage of the fly.

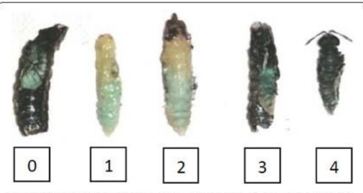

Fig. 1 Photographic-development stage score of the non-emerged flies. 0 = absence of formation, 1 = anatomical formation of the fly without pigmentation, 2 = presence of the fly with pigmented eyes, 3 = fly with the pigmented thorax, 4 = presence of an anatomically complete fly

Statistical analysis

The statistical analysis was performed using the IBM SPSS Statistics software (V27.0.0). The normality or non-normality distribution of the collected data was determined by Shapiro-Wilk test. The experimental unit for all the analysed parameters for the larval, prepupal, pupal and adult stages was the box. On the contrary, the fly represented the experimental unit for the adult ER. Data related to the GA substrate were excluded from the statistical analysis as it was considered as the environmental control only. The larval weights were analysed by fitting a generalized linear mixed model that allowed them to depend on two fixed effects (diet and time [plus, accordingly, their interaction]) through a gamma probability distribution with a nonlinear link function (log). The replicate was included as a random effect to account for repeated measurements on the same box. The interactions between the levels of the fixed factors were evaluated by means of pairwise contrasts.

One-way ANOVA (post-hoc test: Tukey) was, instead, used to compare the L-P phase and the larval survival among the experimental diets. Differently, prepupae and pupae data were tested by Kruskal-Wallis test (post-hoc test: Dunn's Multiple Comparison Test). Finally, the adult stage data were analysed by fitting either a general or generalized linear mixed model, with the only exception of the ER being evaluated with the one-way ANOVA test (post-hoc test: Tukey). In particular, a general linear mixed model allowed the FLW and the EW to depend on two fixed effects (diet and sex [plus, accordingly, their interaction]), while a generalized linear mixed model allowed the WL, P-F duration and FLS to depend on the same fixed effects through a gamma probability distribution with a nonlinear link function (log). In both the statistical models, the replicate was included as a random effect to account for repeated measurements on the same box, and the interactions between the levels of the fixed factors were evaluated by means of pairwise contrasts. A likelihood-ratio test was also performed in case of not significant interaction terms, and, when necessary, a model simplification was applied by removing them from the statistical models. The results were expressed as mean (L-P phase, larval survival, prepupal and pupal weights, and ER) or least square mean (larval weight, FLW, WL, EW, P-F duration

and FLS) and pooled standard error of the mean (SEM). The level of significance considered was < 0.05.

Results

Larval stage

Data recorded for larvae grown on GA diet were consistent with those usually recorded in the experimental facility (data not shown) and allowed to exclude any environmental issues during the trial. At the beginning of the trial, the larvae weight was homogeneous in all the dietary treatments (0.070 ± 0.001; $P > 0.05$). Considering the whole larval stage (Table 2), the CP16 and the CP14 treatments were significantly different (0.170 and 0.163, respectively; $P < 0.001$). On the contrary, the weight of the CP19 larvae was intermediate, being comparable to the experimental diets with 14% and 16% of protein ($P > 0.05$). The CP10 displayed a lower weight when compared to the other dietary treatments ($P < 0.001$). The sampling time influenced the weight of the larvae, as it reflected the physiological larval growth ($P < 0.001$).

Figure 2 shows the interaction between diet and time. The CP14, CP16 and CP19 treatments showed improved growth at 10 and 14 days old (T1 and T2, respectively) in comparison with the CP10 ($P < 0.001$). At 18 days old (T3), the CP19 group showed a lower weight when compared to CP14 larvae (0.223 and 0.238, respectively; $P < 0.01$). The CP10 and the CP16 groups did not differ from the other dietary treatments.

As regards the survival rate, no differences were observed among the experimental diets (CP 10–14-16: 89% and CP19: 90%, $P > 0.05$).

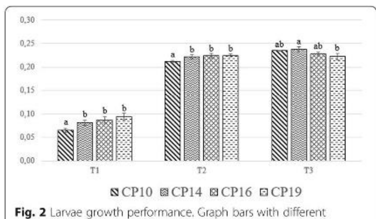

Fig. 2 Larvae growth performance. Graph bars with different superscript (a, b) indicate significant differences among the experimental diets within the sampling time ($P < 0.05$)

Prepupal stage

Figure 3 summarizes the prepupae weight. In particular, the CP10 treatment was statistically different from the CP19 group ($P < 0.01$). On the contrary, the CP14 and the CP10 prepupae weight was intermediate between the lowest and the highest protein level ($P > 0.05$). The GA prepupae appeared less heavy than those of the experimental diets.

Pupal stage

Table 3 shows the effects of the dietary treatments on pupae weight, L-P phase duration and pupae score. In the pupal stage, the CP10 and CP14 treatments showed heavier pupae when compared to the CP16 and CP19 (0.188–0.194 and 0.172–0.174, respectively; $P < 0.001$). The CP16 and CP19 pupae had a similar weight (0.172 and 0.174; $P > 0.05$). On the contrary, the environmental control (GA) displayed the numerically lowest weight.

As regards the L-P phase duration, the larvae that took the longest to pupate were the CP14 (21.80 d), while the shortest time was observed for the CP16 (20.88; $P < 0.001$). The timing of the CP19 treatment was comparable to the CP10 and CP14 groups ($P > 0.05$), while it was statistically different from the CP16 treatment ($P < 0.001$). The GA insects showed a L-P duration time of 16.43 d, thus being apparently shorter than the ones reported in the PSPID.

All the development scores applied to the moribund pupae were close to 4 and no significant differences between the dietary treatments were recorded ($P > 0.05$).

Adult stage

The effects of the dietary treatments and sex on the adult stage parameters are reported in Tables 4 and 5. No differences among the diets were reported for FLW, WR and EW ($P > 0.05$, Table 4). On the contrary, the sex influenced the weight of the exuvia, which was greater in the females than the males ($P < 0.01$, Table 4). The P-F duration time was not affected by the dietary treatments, the sex and the interaction between the two factors ($P > 0.05$; Table 5). Fly sex had an effect on the FLS ($P < 0.05$, Table 5). In particular, the males lived almost a day longer than the females ($P < 0.01$). The CP19 female flies also showed a longer lifespan when compared to the females from the other groups ($P < 0.01$, Fig. 4). Regarding ER, no differences were observed

Table 2 Effects of diet, time and interaction between diet and time on larvae weight (1 larva/weight)

Parameter	Diet (D)				Time (T)			SEM		*P*-value		
	CP10	CP14	CP16	CP19	10 d	14 d	18 d	D	T	D	T	DxT
Weight	0.148[c]	0.163[b]	0.170[a]	0.168[ab]	0.083[c]	0.221[b]	0.231[a]	0.004	0.001	0.001	0.001	0.001

Legend: *CP10* 10% CP diet, *CP14* 14% CP diet, *CP16* 16% CP diet, *CP19* 19% CP diet, *GA* Gainesville diet, *d* days old, *SEM* standard error of the mean.
Means with different superscript letters (a, b, c) within the same row differ significantly ($P < 0.05$)

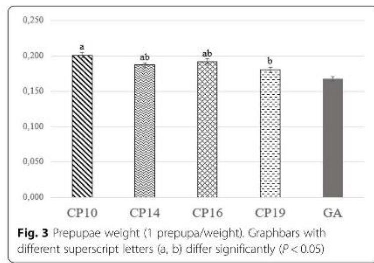

Fig. 3 Prepupae weight (1 prepupa/weight). Graphbars with different superscript letters (a, b) differ significantly ($P < 0.05$)

among the experimental diets ($P > 0.05$). Moreover, the ER displayed by flies reared on experimental diets were very low when compared to the ones reared on the GA diet, despite being in the same environmental conditions (CP10 37.1%, CP14 35.7%, CP16 25%, CP19 35% and GA 86,4%).

Means with different superscript letters (a, b, c) within the same row differ significantly ($P < 0.05$).

Gainesville diet

Data regarding the larva, pupa and adult parameters recorded for the GA are summarized in Table 6.

Discussion

Larval stage

To the author's knowledge, the present study is the first to evaluate the effects of isoenergetic diets with increasing levels of protein on the BSF larva development and flies-related parameters. The BSF is one of the most promising insect species for feed production given its ability to convert waste [3]. However, determining the protein requirement can improve its efficiency and guarantee the optimization of the waste reduction. Handling has been reported to affect either the weight or the survival of the larvae [23]. However, since all the treatments in this experimental trial have undergone the same handling procedures, the impact derived from the manipulation of the larvae can be reliably excluded. The data related to the GA treatment fell within the typical ranges for each development stage, thus making possible to state that no environmental effects altered the herein obtained results. During the whole larval development, the protein level that gave the best result in terms of growth was the 16% on the DM. This result is in agreement with that obtained by Lalander et al. [8] on the larvae reared on poultry feed with a protein content of 17.3% DM, but not with the weight of those raised on primary sludge containing the 16.9% CP on DM. In particular, the average weight of the larvae at 18–20 days of age grown on the poultry feed lied in a range between 200 to 250 mg per larva, while that of the larvae grown on the primary sludge was in 100–150 mg range [8]. Considering the limited protein variation between the two diets, it is presumed that the difference in weight is not due to the protein content, but to the other macronutrients.

At 10 and 14 days of age, the CP10 larvae showed the lowest weight when compared to the other experimental treatments. The CP10 larvae weight (10 days old) was in agreement with that recorded by Meneguz et al. [6] on the vegetables and fruit (VEGFRU) substrate with 11.9% DM of protein level (about 60 mg/larva). On the contrary, at 14 days old, the CP10 larvae weight was almost twice that was recorded on the VEGFRU by the same authors. Considering 18-day-old larvae, the protein level that gave the best result in terms of weight was the one containing 14% of protein on DM (238 mg/larva). Differently, Lalander et al. [8] observed the lowest larvae growth (0–50 mg/larva) in a substrate with a protein level of 14.7% on DM. The results heterogeneity herein reported may be related to the type of supplied protein or the substrate texture. Since experimental diets with higher protein levels performed better in the first two growth periods, while the opposite trend was observed in the third one, it is possible to state that the protein level can be reduced in the last stages of larval development without affecting the growth performance. This condition, from a production point of view, allows the use of waste with a lower protein content in instars close to the prepupae. Given the reduced weight gain between 14 and 18 days of age, this moment can be considered the plateau phase. As a consequence, in terms of meal production, the 14th day of larvae life could be considered optimal. To reach this development stage with the best performance it is recommended to use protein levels equal to or greater than 14% on DM.

Table 3 Effects of the dietary treatments on pupae weight, larva-pupal phase duration and pupae score

Parameters	Dietary treatments				SEM	*P* -value	GA
	CP10	CP14	CP16	CP19			
Weight, g	0.188^{a}	0.194^{a}	0.172^{b}	0.174^{b}	0.004	0.000	0.154
L-P duration, d	21.11^{bc}	21.80^{a}	20.88^{c}	21.57^{ab}	0.104	0.000	16.43
Score	3.98	3.98	3.95	3.99	0.206	0.206	3.80

Legend: *L-P* larva-pupal phase duration (n° of days), *CP10* 10% CP diet, *CP14* 14% CP diet, *CP16* 16% CP diet, *CP19* 19% CP diet, *GA* Gainesville diet.
Means with different superscript letters (a, b, c) within the same row differ significantly ($P < 0.05$)

Table 4 Effects of diet and sex on the fly live weight, fly weight reduction and exuvia weight

Parameters	Diet (D)				Sex (S)		SEM		*P*-value	
	CP10	CP14	CP16	CP19	F	M	D	S	D	S
FLW	0.126	0.139	0.134	0.124	0.140	0.121	0.010	0.007	0.647	0.076
WR	48.87	48.16	49.27	50.24	47.34	48.86	3.031	1.246	0.155	0.068
EW	0.023	0.023	0.024	0.024	0.025	0.022	0.001	0.001	0.303	0.001

Legend: *CP10* 10% CP diet, *CP14* 14% CP diet, *CP16* 16% CP diet, *CP19* 19% CP diet, *GA* Gainesville diet, *FLW* fly live weight, *WR* fly weight reduction, *EW* exuvia weight, *SEM* standard error of the mean

The larval survival rate was high in every treatment, being also greater than that observed by Oonincx et al. [11] in diets with different C:P ratios (72–86%) and Cammack et al. [10] in P:C balanced diet (57–62%). On the contrary, the rate registered in the present study was in agreement with that of Chia et al. [23] on brewing-waste reared larvae. A reduced mortality rate means that the compositions of all diets met the minimum larval nutrient and physical requirements.

Prepupal stage

The weight of the prepupae ranged from 0.180 g to 0.201 g was globally higher than that recorded in Cammack et al. [10] study (range: 0.080–0.110 g). Furthermore, in their study, the 70% humidity control diet performed better than the others with different C:P ratios and moisture content [10]. This result is in contrast to what was found in the present study, in which the GA control (30% of DM) showed the lowest weights. The data obtained from the prepupae of the CP16 treatment was slightly less than those of the Lalander et al. [8] research, where the BSF larvae were fed poultry feed-based diet (0.251 g). The prepupae of primary sludge treatment, as well as the larvae, showed a different weight when related to the results of the present study. The CP16 prepupae performed better even compared to the weight of the prepupae obtained by Spranghers et al. [5] on restaurant waste with a CP percentage of 15.7%. Since similar protein levels were not accompanied by similar results, it may be argued that the diversity of raw materials used in the experimental diets have a major influence, and that semi-purified ingredients would have been more easily metabolized and, consequently, assimilated. The CP10 treatment reported the highest values, with statistical differences against the CP19 diet. In the prepupae phase, the macronutrient *par excellence* that guarantees the reserve for the non-feeding phase is the lipid. For this reason, in the last stages of the mobile period, a reduction in the protein content may not cause any negative consequences on BSF growth.

Pupal stage

In the pupal stage, even in the last instar larvae and prepupae, the best performance in terms of weight were observed in the experimental diets with lower protein levels. Considering the time needed for larvae to reach pupae stage (L-P duration), all the experimental treatments showed values close or higher than 21 d, while GA larvae took about 16.5 d. It has been reported that the larvae development time is usually affected by the quantity of macronutrients in the diets, specifically lipids [24]. If the substrate is low in fat, the larvae need more time to acquire their fat body and, consequently, take longer to complete their growth [24]. This last statement agreed with our results, since the experimental diets were characterized by a lipid content that varied from 0.92 to 1.27% (as is), while the GA showed an EE value of 2.22% (as is). The absence of isolipidic diets may represent a limitation of the present study. However, at the same time, this gap represents the importance of determining the minimum macronutrient requirements in order to guarantee improved performance in all the phases of the BSF life cycle.

Adult stage

Data related to the life parameters may have been influenced by the rearing box. In particular, the flight space was reduced compared to the cages one. Moreover, it is possible to hypothesize a reduced energy consumption compared to the breeding conditions. Only the sex influenced the size of the flies and the exuvia weight. Specifically, the males tended to be smaller than the females, thus being in agreement with Jones and Tomberlin [25], which observed that, in their colony, the weight of the

Table 5 Effects of diet, sex and interaction between diet and sex on pupa-fly duration time and fly lifespan

Parameters	Diet (D)				Sex (S)		SEM		*P*-value		
	CP10	CP14	CP16	CP19	F	M	D	S	D	S	D x S
P-F	7.04	7.02	7.29	6.72	7.19	6.84	0.281	0.218	0.090	0.248	0.365
FLS	9.01	8.43	8.45	9.36	8.36	9.27	0.342	0.314	0.211	0.035	0.001

Legend: *CP10* 10% CP substrate, *CP14* 14% CP substrate, *CP16* 16% CP substrate, *CP19* 19% CP substrate, *GA* Gainesville diet, *P-F* pupa-fly duration time, *FLS* fly lifespan, *SEM* standard error of the mean

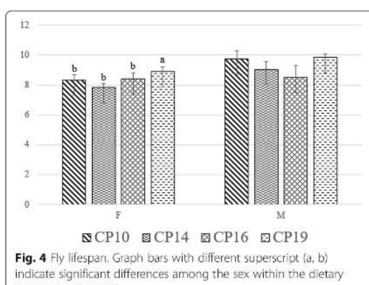

Fig. 4 Fly lifespan. Graph bars with different superscript (a, b) indicate significant differences among the sex within the dietary treatments ($P < 0.05$)

males was constantly lower when compared to the females. The EW displayed the same results in relation to the sex, as it was significantly greater in the females than the males. The WR was not affected by the dietary treatments, but it showed a tendency to significance in relation to the sex as well. In particular, the males tended to show higher weight loss due to the longer lifespan when compared to females. Furthermore, females have been reported to display a highest quantity of dry matter, thus reliably explaining the reduced weight loss [26]. Gao et al. [27] also observed a longer lifespan in males than females (10.32 and 9.31 d, respectively). The females accumulate higher energy reserve than males for the eggs production [28]. Since in the present trial the mating was not possible and the females lived less than the males, it is possible to speculate that the energy reserve accumulated for reproductive purposes – in absence of reproduction activity – was not channelled for other uses.

Table 6 Larva, pupa and adult parameters recorded for the environmental control

Larvae (day old)	Weight, g
6	0.070
10	0.089
14	0.204
18	0.167
Pupae	
Weight, g	0.154
L-P duration, d	16.43
Score	3.80
Adult	
WR, %	49.51
FLW, g	0.103
EW, g	0.019
P-F duration, d	7.55
FLS, d	8.10

Legend: *L-P* larva-pupal phase duration, *WR* fly weight reduction, *FLW* fly live weight, *EW* exuvia weight, *P-F* pupa-fly duration time, *FLS* fly lifespan

The females of the diet with the highest protein level lived longer than the others, thus making reasonable to hypothesize that the protein accumulated in the larval stage, unlike the energy, may have an effect on the adult life duration.

The total duration in days of the period between the pupal stage and the adult emergence was studied by Barros-Cordeiro et al. [22]. These authors demonstrated that under controlled conditions (27 ± 1.0 °C, 60 ± 10% RH, 12:12 L:D), the P-F duration is 8 d. In the present study, the numerically shortest period (6.7 d) was recorded in the experimental treatment with the highest protein level (CP19). However, the differences between the treatments that took less time for the adult emergence and that took the longest (CP16) did not exceed 1 d. The difference in the results obtained from the two studies may derive from the adoption of different temperature and photoperiod conditions. Indeed, an increase in the temperature of the pupal mass can cause a pupal phase reduction, while an inadequate photoperiod can lengthen it [29].

The emergence rate was found to be extremely low in all the experimental groups. Considering that the dissected moribund pupae were at the most advanced development stage, it is possible to speculate that the reduced lipid intake of the practical semi-purified diets allowed the development of the fly in the puparium, but it was not sufficient to guarantee its emergence. Indeed, the experimental diets contained almost half of the lipid content (in percentage) compared to GA (CP10 was 43% less, CP14 52%, CP16 56% and CP19 58%). In Diptera, an eclosion hormone has been reported to influence the adult emergence [30]. Generally, the arthropod hormones involved in moulting and in other processes belong to the steroid class and are defined ecdysteroids [31]. Since insects cannot synthesize cholesterol de novo from acetate, they are dependent on the cholesterol ingested during the nutritional phase [31]. For this reason, the lipid component has another important function in the emergence moment. In particular, the fly must have the force to open the operculum of the puparium and crawl out of it [30]. Moreover, in nature the black soldier fly prefers the underground as pupariation site, and, consequently, it needs to dig the way upward through the soil [30]. Based on the above-reported argument, it is possible to hypothesize that, if the fly does not have an optimal quick-use lipid reserve, emerging may be hampered. This condition implies that, although a substrate guarantees good performance in the larval

stage, it may generate disorders in the other phases of the life cycle. For this reason, the determination of the larvae nutritional requirements and the evaluation of the effects in prepupae, pupae and adults are necessary to formulate waste-based diets that do not lead to a deficit within the whole production cycle.

Conclusion

The successful development of the BSF larvae in the PSPID demonstrates that they can be used for the determination of the nutritional requirements. Based on the results herein obtained, the optimal protein level for the maximum larvae size in the entire larvae stage might be considered 16% on DM, while at 18 days old the 14% on DM. Given the results obtained in the 18-day-old larvae, in the prepupa and pupa stages, it will be interesting to evaluate the possibility of using substrates with a lower protein content in the last development phases. The low adult emergence rate in PSPID diets may not be attributable to the protein, thus making the conduction of further researches mandatory in order to determine which nutrient deficiency leads to this outcome. Finally, this work lays the foundation for research in the evaluation of substrate qualitative assessment not only during the larval stage, but also in all BSF life history traits.

Abbreviations

BSF: Black soldier fly; P: Protein; C: Carbohydrate; GA: Gainesville diet; DM: Dry matter; CP: Crude protein; EE: Ether extract; GE: Gross energy; aNDFom: Amylase neutral detergent fiber organic matter; NSC: Non-structural carbohydrates; SEM: Standard error of the mean; L-P: Larva-pupal; FLW: Fly live weight; DFW: Dead fly weight; EW: Exuvia weight; P-F: Pupa-fly; FLS: Fly lifespan; ER: Emergence rate

Authors' contributions

SBO, IB and LG conceived and designed the experiment, performed the trial and collected the experimental data. SBO and IB analysed the data. SBO carried out the chemical analyses. SBO wrote the first draft of the manuscript. All the authors critically reviewed the manuscript for its intellectual content and gave their approval for the final version to be published.

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Smetana S, Schmitt E, Mathys A. Sustainable use of *Hermetia illucens* insect biomass for feed and food: attributional and consequential life cycle assessment. Resour Conserv Recycl. 2019;144:285–96. https://doi.org/10.1016/j.resconrec.2019.01.042.
2. Gasco L, Biancarosa I, Liland NS. From waste to feed: a review of recent knowledge on insects as producers of protein and fat for animal feeds. Curr Opin Green Sustain Chem. 2020;6:67–79. https://doi.org/10.1016/j.cogsc.2020.03.003.
3. Raksasata R, Lima JW, Kiatkittipong W, Kiatkittipong K, Ho JC, Lame MK, et al. A review of organic waste enrichment for inducing palatability of black soldier fly larvae: wastes to valuable resources. Environ Pollut. 2020;267:115488. https://doi.org/10.1016/j.envpol.2020.115488.
4. Wang YS, Shelomi M. Review of black soldier Fly (*Hermetia illucens*) as animal feed and human. Food foods 2017. 2017;6(10). https://doi.org/10.3390/foods6100091.
5. Spranghers T, Ottoboni M, Klootwijk C, Ovyn A, Deboosere S, De Meulenaer B, et al. Nutritional composition of black soldier fly (*Hermetia illucens*) prepupae reared on different organic waste substrates. J Sci Food Agr. 2017;97(8):2594–600. https://doi.org/10.1002/jsfa.8081.
6. Meneguz M, Schiavone A, Gai F, Dama A, Lussiana C, Renna M, et al. Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (*Hermetia illucens*) larvae. J Sci Food Agr. 2018;98(15):5776–84. https://doi.org/10.1002/jsfa.9127.
7. Manurung R, Supriatna A, Esyanthi RR, Ramadhani EP. Bioconversion of Rice straw waste by black soldier fly larvae (*Hermetia illucens* L.): optimal feed rate for biomass production. J Entomol Zool Stud. 2016;4:1036–41.
8. Lalander C, Diener S, Zurbrügg C, Vinnerås B. Effects of feedstock on larval development and process efficiency in waste treatment with black soldier fly (*Hermetia illucens*). J Clean Prod. 2019;208:211–9. https://doi.org/10.1016/j.jclepro.2018.10.017.
9. Barragan-Fonseca KB, Gort G, Dicke M, Van Loon JJA. Effects of dietary protein and carbohydrate on life-history traits and body protein and fat contents of the black soldier fly *Hermetia illucens*. Physiol Entomol. 2019;44(2):148–59. https://doi.org/10.1111/phen.12285.
10. Cammack JA, Tomberlin JK. The impact of diet protein and carbohydrate on select life-history traits of the black soldier fly *Hermetia illucens* (L.) (*Diptera: Stratiomyidae*). Insects. 2017;8:56. https://doi.org/10.3390/insects8020056.
11. Oonincx DGAB, van Broekhoven S, van Huis A, van Loon JJA. Feed conversion, survival and development, and composition of four insect species on diets composed of food by-products. PLoS One. 2015;10(12):1–7. https://doi.org/10.1371/journal.pone.0144601.
12. Scott HM, Moellek MW, Hinnees SW. Studies with purified diets. Poult Sci J. 1957;36(5):1000–2. https://doi.org/10.3181/00379727-95-23346.
13. Goettsch M. Comparative protein requirement of the rat and mouse for growth, reproduction and lactation using casein diets. Nutr J. 1959;70(3):307–12. https://doi.org/10.1093/jn/70.3.307.
14. Oltjen RR, Sirny RJ, Tillman AD. Purified diet studies with sheep. J Anim Sci. 1962;21(2):277–83. https://doi.org/10.2527/jas1962.212277x.
15. Kim KI, Kayes TB, Amundson CH. Purified diet development and re-evaluation of the dietary protein requirement of fingerling rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 1991;96(1):57–67. https://doi.org/10.1016/0044-8486(91)90139-X.
16. Lagoc J. Aqua Farm News. 1989;6:1–7.
17. Hogsette JA. New diets for production of house flies and stable flies (*Diptera: Muscidae*) in the laboratory. J Econ Entomol. 1992;85(6):2291–4. https://doi.org/10.1093/jee/85.6.2291.
18. AOAC International. Official methods of analysis of AOAC international. 17th ed. Association of Official Analytical Chemists: Gaithersburg; 2000.
19. AOAC International. Official methods of analysis of AOAC International. 17th; 2nd revision. Gaithersburg Association of Official Analytical Chemists; 2003.
20. Mertens DR. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. J AOAC Int. 2002;85(6):1217–39.
21. Holmes LA, Vanlaerhoven SL, Tomberlin JK. Substrate effect on pupation and adult emergence of *Hermetia illucens* (*Diptera: Stratiomyidae*). Environ Entomol. 2013;42(2):370–4. https://doi.org/10.1603/EN12255.

22. Barros-Cordeiro KB, Nair Báo S, Pujol-Luz JR. Intra-puparial development of the black soldier-fly, *Hermetia illucens*. Insect Sci. 2014;14(83):1–10. https://doi.org/10.1093/jis/14.1.83.
23. Chia S, Tanga CM, Osuga IM, Mohamed SA, Khamis FM, Salifu D, et al. Effects of waste stream combinations from brewing industry on performance of black soldier Fly, *Hermetia illucens* (*Diptera: Stratiomyidae*). Zool Sci. 2018;6:1–26. https://doi.org/10.7717/peerj.5885.
24. Nguyen TTX, Tomberlin JK, Vanlaerhoven S. Influence of resources on *Hermetia illucens* (Diptera: *Stratiomyidae*) larval development. J Med Entomol. 2013;50(4):898–906. https://doi.org/10.1603/me12260.
25. Jones BM, Tomberlin JK. Effects of the adult body size on mating success of the black soldier fly, *Hermetia illucens* (L.) (*Diptera: Stratiomydae*). J Insects Food Feed. 2021;7(1):5–20. https://doi.org/10.3920/JIFF2020.0001.
26. Liu X, Chen X, Wang H, Yang Q, ur Rehmen K, Li W, et al. Dynamic changes of nutrient composition throughout the entire life cycle of black soldier fly. PLoS ONE. 2017;12(8):e0182601. https://doi.org/10.1371/journal.pone.0182601.
27. Gao Z, Wang W, Lu X, Zhu F, Liu W, Wang X, et al. Bioconversion performance and life table of black soldier fly (*Hermetia illucens*) on fermented maize straw. J Clean Prod. 2019;230:974–80. https://doi.org/10.1016/j.jclepro.2019.05.074.
28. Tomberlin JK, Adler PH, Myers HM. Development of the black soldier fly *(Diptera: Stratiomyidae)* in relation to temperature. Environ Entomol. 2009;38(3):930–4. https://doi.org/10.1603/022.038.0347.
29. Ma T, Huang J, Wang JF. Study on the pupal morphogenesis of *Chrysomya rufifacies* (Macquart)(Diptera: *Calliphoridae*) for postmortem interval estimation. Forensic Sci Int. 2015;253:88–93. https://doi.org/10.1016/j.forsciint.2015.06.005.
30. Denlinger DL, Zdarek J. Metamorphosis behavior of flies. Annu Rev Entomol. 1994;39(1):243–66. https://doi.org/10.1146/annurev.en.39.010194.001331.
31. Spindles KD, Hönl C, Tremmel C, Braun S, Ruff H, Spindles-Barth M. Ecdysteroid hormone action. Cell Mol Life Sci. 2009;66(24):3837–50. https://doi.org/10.1007/s00018-009-0112-5.

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Determination of lipid requirements in black soldier fly through semi-purified diets

S. Bellezza Oddon, I. Biasato✉, A. Resconi & L. Gasco

The insect market is still far from an effective upscale and, to achieve this goal, it is necessary to know the BSF dietary requirements for the production maximization. Worldwide, given the waste variability, is not always easy to identify the optimal waste-based mixture that can allow to reach the best production, in terms of quantity and quality. Due this reason, nutritional need ranges are the basic knowledge, affordable for everyone, to increase the profitability of the insect farming. The study aims to evaluate the effects of 6 semi-purified, isonitrogenous and isoenergetic diets (SPII) with increasing lipid levels (1%, L1; 1.5%, L1.5; 2.5%, L2.5; 3.5% L3.5; 4.5%, L4.5) on BSF life history traits (6 replicates/treatment and 100 larvae/replicate). The Gainesville diet was used as environmental control. Considering the whole larval stage, 4.5% lipid level guarantees better performance when compared to content lower than 2.5%. The L4.5 10-day-old larvae yielded greater when compared to the other dietary treatments. At 14 and 18 days of age, the larvae of the groups above 2.5% performed better than L1, while the L1.5 showed intermediate results. Lipid levels below 1.5% on DM, when compared to 4.5%, resulted in a smaller prepupa and pupa size. The results obtained on the adult stage do not allow the identification of a lipid levels ideal range, as in the larval stage. In conclusion, in the whole larval stage and in prepupae/pupae phases, lipid percentage lower than (or equal to) 1% have a negative effect on growth. Other research will be needed in order to evaluate lipid levels above 4.5% on DM.

Recently, insects are receiving more and more attention as farmed animals, both for feed and food production. Their capacity to be reared using less space and water, their low greenhouse gases emissions, and their high protein content make them a valuable and sustainable choice as alternative sources of protein[1,2]. Out of the 7 species allowed to be farmed for poultry, swine and aquaculture feed production, the black soldier fly (BSF, *Hermetia illucens*) is the one who is receiving the most attention from both the scientific community and the industry sector[3]. Its cosmopolitan distribution, the ability to feed on a large variety of organic substrates, the relative ease of rearing compared to other insect species, and its high feed conversion rate make the BSF one the most important candidates as novel protein source for the feed sector[4–6]. Black soldier fly larvae (BSFL) can, indeed, be reared on waste that would be otherwise sent to landfills or, in the best-case scenario, composting facilities[7,8]. Composting, however, would not valorise the remaining nutrient content of the organic material that is used for it. The BSFL breeding, on the other hand, can turn waste into valuable protein for the feed industry[9,10]. The insect protein industry is currently facing two main challenges that still prevent it to become competitive on the market: the need to upscale and the regulatory barriers[11]. Despite the recent authorization of BSFL-based protein feed for poultry and pigs (Reg. (EU) 2021/1372) having allowed to significantly extend the legislative framework, the insect market is still far from an effective upscale[12–14].

The necessity of insect industrial production maximization stimulates the scientific community to pursue its efforts on BSFL rearing requirements determination, and the knowledge of bred-animals nutritional needs is the farming optimization starting point. This has been known for decades for the major animal species reared for human consumption, but, in spite of the high number of studies done on BSF, there is still a very limited knowledge about the dietary requirements for the correct development of the larvae and for their metamorphosis into imagos (adult stage[15–17]). The scientific works currently available in literature focus on protein and carbohydrate needs, as well as the relationship between the two[18,19]. Proteins represent an important nutritional source, since they have a plastic and growth-related function, while carbohydrates are an energetic font. Since the holometabolous insect (they present a larval stage and an adult stage with clear differences: feeding habits, morphology) alternates in different development phase feeding and non-feeding periods, the fat body cells are

Department of Agricultural, Forest and Food Sciences, University of Turin, Turin, Italy. ✉email: ilaria.biasato@unito.it

Parameter	Diet (D)					Time (T)			SEM		p-value		
	L1	L1.5	L2.5	L3.5	L4.5	T1	T2	T3	D	T	D	T	DxT
Weight (g)	0.161^{d}	0.167^{cd}	0.172^{bc}	0.176^{ab}	0.180^{a}	0.105^{c}	0.201^{b}	0.236^{a}	0.003	0.002	0.001	0.001	0.001

Table 1. Effects of the dietary treatment, time and interaction between dietary treatment and time on individual larval weight. L1 EE 1% diet, L1.5 EE 1.5% diet, L2.5 EE 2.5% diet, L3.5 EE 3.5% diet, L4.5 EE 4.5% diet, T1 10-day-old, T2 14-day-old, T3 18-day-old, SEM standard error of the mean. Means with different superscript letters (a, b, c) within the same row differ significantly ($p<0.05$).

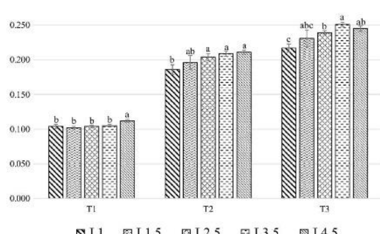

Figure 1. Effects of the interaction between dietary treatment and time on larva growth. *L1* EE 1% diet, *L1.5* EE 1.5% diet, *L2.5* EE 2.5% diet, *L3.5* EE 3.5% diet, *L4.5* EE 4.5% diet, *GA* Gainesville diet, *T1* 10 day old, *T2* 14 day old, *T3* 18 day old. Means with different superscript letters (a, b, c) within the same row differ significantly ($p<0.05$). The standard error is represented by the error bars.

necessary for their survival during starvation[20]. During the feeding-phase, the carbohydrates surplus is converted in lipid reserve thanks to the insulin activity[1,21].

The lipid component has another important function during the Diptera life cycle. In particular, the moulting process begins from the ecdysteroids, which are hormones that belong to the steroid class and whose synthesis occurs from the cholesterol ingested in the feeding phase[22]. In a recent paper by Bellezza Oddon et al.[23], a reduced fly emergence rate was observed in the experimental diets with halved lipid content (ether extract [EE] as is: < 1.27) when compared to the GA diet (EE as is: 2.22). In arthropod, the moult occurs thanks to steroid hormones called ecdysteroids. Insects cannot synthesize cholesterol and, consequently, the feeding-phase is important for its assimilation[24]. Another study reported the important role that lipids play in the development of BSFL, with longer time needed to reach the prepupal stage in diets lower in lipids and vice-versa[25].

Since lipids seems to have an effect on insect physiology, can they also have an influence on growth and to what extent?

Based on the above-reported background, this study aims at assessing the optimal lipid content that can guarantee the greatest development of BSF throughout their entire cycle, from larval stage to pupation and fly emergence by using semi-purified, isonitrogenous and isoenergetic diets.

Results

Larval stage. At 6 days of age, the larvae weight was analogous in all the treatments (0.088 g ± 0.001; $P>0.05$). Table 1 illustrates the effects of the diet and the time on larvae growth. Considering the whole larval stage, the treatment with the highest lipid content (L4.5) showed heavier weight when compared to the L1, L1.5 and L 2.5 diets (0.180 g, 0.161 g, 0.167 g and 0.172 g, respectively; $P<0.01$), while the L3.5 group (0.176 g) displayed an intermediate result between the L2.5 and the L4.5 ($P>0.05$). Time had, naturally, an effect on larvae weight (T1, 10 days old; T2, 14 days old; T3, 18 days old; $P<0.01$). The interaction between diet and time is shown in Fig. 1. At T1, the L4.5 treatment performed better (0.112 g) when compared to the other groups (L1, 0.104 g; L1.5, 0.102 g; L2.5, 0.104 g; L3.5, 0.105 g; $P<0.01$). In the second sampling time (T2), the L2.5 and L3.5 treatments were not different from the L4.5 (0.204 g, 0.209 g and 0.211 g, respectively; $P>0.05$). On the contrary, the L1 larvae showed the worst result (0.186 g; $P<0.01$), while L1.5 did not differ among the groups (0.196 g; $P>0.05$). At T3, the L3.5 group showed higher larvae weight than the L1 and the L2.5 (0.251 g, 0.217 g and 0.239 g, respectively; $P<0.01$), but analogous results in comparison with the L1.5 and the L4.5 treatments (0.231 g and 0.245 g; $P>0.05$). Regarding the survival rate, no differences were observed among the groups (L1, 85%; L1.5, 93%; L2.5, 90%; L3.5, 95%; L4.5, 95%; $P>0.05$).

	Larvae (% on DM)				
Items[a]	L1	L1.5	L2.5	L3.5	L4.5
DM	32.43	30.80	29.76	31.40	30.11
CP	23.11	28.44	30.44	28.28	30.35
EE	47.45	39.39	38.52	37.91	33.21
Ash	5.22	5.61	5.77	5.95	6.16
GE	30.14	29.16	28.95	28.97	29.58

Table 2. Larvae descriptive chemical composition (g/100 g on DM) and gross energy (MJ/kg on DM) expressed on DM. L1 EE 1% diet, L1.5 EE 1.5% diet, L2.5 EE 2.5% diet, L3.5 EE 3.5% diet, L4.5 EE 4.5% diet, DM dry matter, CP crude protein, EE ether extract, GE gross energy. [a]Values are reported as mean of duplicate analyses.

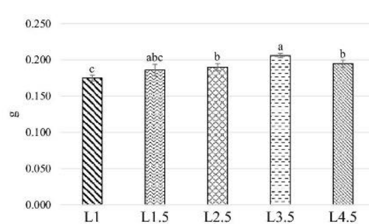

Figure 2. Diet effect on the prepupae weight. *L1* EE 1% diet, *L1.5* EE 1.5% diet, *L2.5* EE 2.5% diet, *L3.5* EE 3.5% diet, *L4.5* EE 4.5% diet, *GA* Gainesville diet. Means with different superscript letters (a, b, c) within the same row differ significantly ($p < 0.05$). The standard error is represented by the error bars.

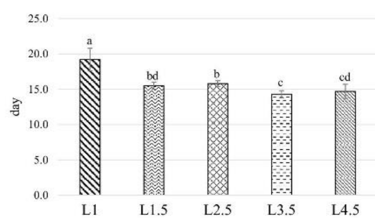

Figure 3. Time necessary to reached the prepupae phase. *L1* EE 1% diet, *L1.5* EE 1.5% diet, *L2.5* EE 2.5% diet, *L3.5* EE 3.5% diet, *L4.5* EE 4.5% diet, *GA* Gainesville diet. Means with different superscript letters (a, b, c) within the same row differ significantly ($p < 0.05$). The standard error is represented by the error bars.

Chemical analysis. The descriptive chemical composition of the larvae is shown in Table 2. The DM ranged from 29.76 to 32.43%. The percentage on the larvae DM of the EE tend to decrease with the increasing of the diet EE level (L1:47.45%, L1.5: 39.39%, L2.5: 38.52%, L3.5: 37.91% and L4.5: 33.21%). The GE values, CP and ash content on DM ranged from 28.97 MJ/kg to 30.14 MJ/kg, from 23.11% to 30.35% and from 1.69 to 1.87%, respectively.

Prepupae. Figure 2 and 3 summarizes the diet effect on prepupae weight and the L-Pp development time. The L1 group showed a lower weight when compared to the L2.5, L3.5 and L4.5 diets (0.175 g vs 0.190, 0.206 and 0.196, respectively; $P < 0.001$), with L3.5 being the one which yielded the heaviest prepupae ($P < 0.001$). The L1.5 treatment was not significantly different from the other experimental diets (0.186 g; $P > 0.05$).

The L1 treatment had the longest development time (19.2 days), while the diet which caused the fastest development was the L3.5 (14.3 days), which was not significantly different from L4.5 (14.7 days; $P > 0.05$). The

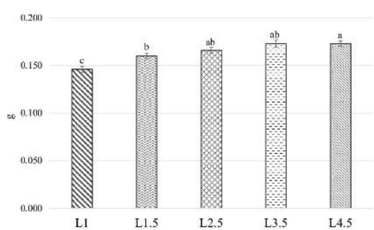

Figure 4. Effect of the diet on the pupae weight. *L1* EE 1% diet, *L1.5* EE 1.5% diet, *L2.5* EE 2.5% diet, *L3.5* EE 3.5% diet, *L4.5* EE 4.5% diet, *GA* Gainesville diet, *T1* 10 day old, *T2* 14 day old, *T3* 18 day old. Means with different superscript letters (a, b, c) within the same row differ significantly ($p<0.05$). The standard error is represented by the error bars.

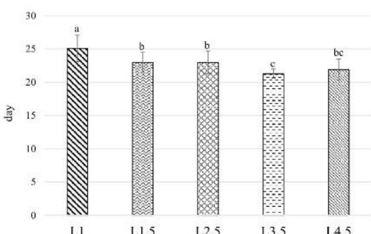

Figure 5. Larva-pupa duration time (day). *L1* EE 1% diet, *L1.5* EE 1.5% diet, *L2.5* EE 2.5% diet, *L3.5* EE 3.5% diet, *L4.5* EE 4.5% diet, *GA* Gainesville diet, *T1* 10 day old, *T2* 14 day old, *T3* 18 day old. Means with different superscript letters (a, b, c) within the same row differ significantly ($p<0.05$). The standard error is represented by the error bars.

L2.5 prepupae showed an intermediate result between the L1 and L3.5 (15.8 days; $P<0.001$). Finally, the L1.5 group (15.5 days) performed similarly when compared to L2.5 and L4.5 ($P>0.05$).

Pupal stage. Data about pupae weight and L–P duration time are illustrated in Figs. 4 and 5. The pupae of the L4.5 diet were heavier than the L1 and L1.5 groups (0.173, 0.146 g and 0.160 g, respectively; $P<0.001$). The L2.5 (0.166 g) and L3.5 (0.173 g) pupae displayed similar weights to L4.5 and L1.5 treatments ($P>0.05$). The L–P time duration showed an opposite trend when compared to the weight, as the L3.5 treatment developed faster than the L1, L1.5 and L2.5 groups (21.3 days, 25.1 days, 23 days and 23 days, respectively; $P<0.01$), but was not significantly different from L4.5 (21.9 days; $P>0.05$). The L4.5 also displayed analogous L–P duration times to those of the L1.5 and L2.5 diets ($P>0.05$), while the L1 group had the slowest development when compared to the other groups ($P<0.01$).

Adult stage. Table 3 illustrates the effect of the diet and the sex on the adult parameters. The longest FLS was recorded in the L2.5 treatment and the shortest in the L1.5 group (8.66 days and 7.90 days, respectively; $P<0.05$), while the L1, L3.5 and L4.5 did not differ from the other experimental diets ($P>0.05$). The FLS was also influenced by the sex, as the males had a longer lifespan when compared to the females ($P<0.001$). The WR was also affected by the treatment and sex. During the life of the flies, the L1, L1.5 and L4.5 showed a greater WR than L3.5 ($P<0.05$), while L2.5 had analogous WR to all the treatments ($P>0.05$). As regards the sex effects on WR, the females lost more weight during the lifetime than the males ($P<0.01$). The FLW and P–F duration time were affected by both the treatment and the sex, while the PW only by sex. The L3.5 FLW was numerically heaviest and significantly different from L1 and L1.5 ($P<0.01$). No differences were observed among the FLW of L1 and L1.5, while the L2.5 and L4.5 only varied from L1 ($P<0.01$). The P–F duration time was shorter in the L1, L1.5 and L2.5 diets when compared to the L4.5 group ($P<0.05$). On the contrary, the L3.5 diet did not differ from the other dietary treatments ($P>0.05$). Considering the sex influence, females took longer time to become

Parameters	Diet (D)					Sex (S)		SEM		p-value	
	L1	L1.5	L2.5	L3.5	L4.5	F	M	D	S	D	S
FLS (day)	8.09^{ab}	7.90^{b}	8.66^{a}	8.31^{ab}	8.28^{ab}	7.48	9.08	0.200	0.115	0.022	0.000
WR (%)	51.46^{a}	52.29^{a}	51.43^{ab}	50.37^{b}	51.67^{a}	52.09	50.80	0.453	0.287	0.039	0.001
FLW (g)	0.082^{c}	0.087^{bc}	0.095^{ab}	0.101^{a}	0.093^{ab}	0.102	0.081	0.003	0.002	0.001	0.000
P–F (day)	6.98^{b}	7.14^{b}	7.11^{b}	7.18^{ab}	7.45^{a}	7.26	7.07	0.063	0.125	0.015	0.016
PW (g)	0.021	0.025	0.024	0.027	0.025	0.026	0.023	0.002	0.001	0.263	0.000

Table 3. Effects of the diet and sex on adult parameters. D diet, S sex, F female, M male, L1 EE 1% diet, L1.5 EE 1.5% diet, L2.5 EE 2.5% diet, L3.5 EE 3.5% diet, L4.5 EE 4.5% diet, SEM standard error of the mean, FLS fly lifespan, WR weight reduction, FLW fly live weight, P–F pupae fly duration time, PW puparium weight. Means with different superscript letters (a, b, c) within the same row differ significantly ($p < 0.05$).

Parameters	Diet					SEM	p-value
	L1	L1.5	L2.5	L3.5	L4.5		
ER	87.70	88.83	94.16	95.99	95.76	3.154	0.083
SR	0.77	0.93	0.95	0.86	0.85	0.123	0.734

Table 4. Effect of the diet on the emergence rate and sex ratio. L1 EE 1% diet, L1.5 EE 1.5% diet, L2.5 EE 2.5% diet, L3.5 EE 3.5% diet, L4.5 EE 4.5% diet, ER emergence rate, SR sex ratio, SEM standard error of the mean.

flies and displayed a greater FLW and PW when compared to the males ($P < 0.05$). Finally, the ER and SR were not influenced by the dietary treatments (Table 4; $P > 0.05$).

Discussion

Larval stage. In recent years, research has started to focus the attention on the BSF nutritional requirements determination. In particular, studies related to protein, energy and carbohydrate needs have recently been carried out[23,26,27]. The knowledge on BSF nutrient requirements is still limited, even if the great number of scientific papers published on its life cycle and its use as feed ingredient[28–30]. In insects with a non-feeding adult stage, the ratio between protein and carbohydrate plays a significant role for the survival, growth and reproduction[19]. On the other side, lipids are an important source of energy during metamorphosis[30], and, to the author's knowledge, data about the effect of the substrate lipid content on BSF life history traits are absent in literature.

Since the weight gain and development time of the larvae reared on the GA diet presented a growth pattern in line with those regularly recorded in the experimental centre colony (data not shown), it can be excluded that some environmental factors altered the results of the trial.

Overall, the difference between the treatments with the lowest and highest lipid level underlines that not only the protein and the carbohydrate content have an effect on the growth performance of the larvae. Despite Barragan-Fonseca et al.[19] having observed that the larvae yield was greater in the diets with a high amount of carbohydrate (NSC percentages on DM: 35, 45 and 55), in the present study, the better growth was recorded in the treatment with the lowest carbohydrate content (76.33% of NSC on DM) when compared to the other treatments (NSC range: from 77.87 to 80.17% on DM). Furthermore, since the protein level was equal in all the experimental diets, it may be possible to attribute the difference in growth between L1 and L4.5 to the lipid effect.

Considering larval stage at different times, at T1 L4.5 yielded the biggest larvae when compared to the other dietary treatments. At 14 and 18 days of age, the larvae of the groups above 2.5% performed better than L1, while the L1.5 showed intermediate results. Since the L1.5 treatment showed great variability, no difference was observed among the L1.5 treatment and the others. The human error was excluded as an influencing factor as the standard error value maintained high at T2 and T3, as well as for the prepupae weight. From a nutritional point of view (lipid and protein content), the L3.5 dietary treatment is partially comparable to the ED_1 diet formulated by Georgescu et al.[26]. Already at 10 days of age, the L3.5 larvae were smaller than ED_1 (0.105 mg and about 0.162 mg, respectively). The difference between the two studies may be due to the different carbohydrate percentage or to the quality of the supplied macronutrients. In particular, in semi-purified diets macronutrients are present almost in purity when compared to the ingredients of GA and soybean meal used by Georgescu et al.[26]. Finally, considering that the marked difference recorded at T1 among the treatment with the highest lipid content and the others was lost at T2 and T3, it is possible to hypothesize that the lipid requirement is highest in early larval stages. A similar trend was observed in the results from the Bellezza Oddon et al.[23] study, where larval weight at 10 days was highest in the treatment with the highest percentage of CP (19% of DM), while at 18 days the treatment that yielded the best results in terms of weight was 14% CP on DM. This can point out that in early stages of development larvae have greater needs in terms of nutrients and during the latest stages those needs diminish. In the present trial, no differences can be observed between 2.5% and 4.5% at 14 and 18 days of age. In the light of this result, it could be interesting, in an industrial rearing facility, to tailor specific diets for the first stages of development that are rich in nutrients, and use substrates that are less nutritious for the late stages. Similar results were found by Meneguz et al.[32] between larvae reared on brewery by-products (BRE) and

on winery by-products (WIN). The BRE diet had higher EE and CP (respectively 86.7 and 200.5 g/kg of DM) when compared to the WIN diet (79.0 and 117.4 g/kg of DM). At 10 days of age, the BRE larvae yielded the best results than WIN (92 mg and 17 mg, respectively), while at the end of their development there was no significant difference between the two treatments. Also in this case, a substrate richer in nutrients impacted significantly on BSFL early stages growth, while differences in the later instars are less evident.

Larval survival among the treatments was not different, and the percentages of survivorship from larvae to prepupae are similar to the values found by Tomberlin et al.[33].

The lipid composition of the L1.5, L2.5 and L3.5 larvae is in agreement with the range from 37 to 41% of EE on DM observed by Georgescu et al.[26] in the diet with about 3% of EE and 13.5% of CP. On the contrary, L1 had the highest fat content (47.5% on DM) and L4.5 the lowest one (33.2% on DM). The larvae fat fraction decreases with increasing the lipid content of the diets. In particular, when compared to the L4.5 larvae, the L1 were composed of 30% more lipids. The presence of a greater amount of fat in the larvae with a low-fat diet can be explained by the transformation of the excess carbohydrates into a lipid reserve[34]. To maintain diets isoenergetic, the amount of carbohydrates was reduced as the lipid level increased. Cohn et al.[27] evaluated the effect of different carbohydrates groups on the proximate composition of the larvae and observed that, in diets containing wheat starch, the lipid percentage of the larvae was higher than the corn starch (over 45% on DM). Regarding the protein content of the larvae, all the dietary treatment showed lower values (≤ 30% on DM) when compared to those observed by Gold et al.[35] [> 33% on DM]) in waste-based diets with 14% of CP and over 5% of EE on DM. Although the protein level of the experimental diets was the same, a difference was found in the protein composition of the larvae. In particular, the difference in percentage between the treatments with the highest (L4.5) and lowest (L1) values was 7.24%. Considering the same protein level in the diets and the difference in the composition of the larvae, it is possible to hypothesize that the metabolization of the protein content is a complex physiological mechanism and may derive from macroelement interactions. As regard the larvae ash content, although the experimental diet had the same percentage, in the larvae the quantity of minerals increases with increasing the lipid level. This nutritional fraction of the diet is currently little-studied, but the metabolization of micronutrients can have an influence on the quality of the product derived from the BSF larvae breeding.

Prepupal stage. If the variability of the L1.5 is considered as in larval stage, the L1 treatment yielded the smallest prepupae among all the treatments. In addition, the group with the lowest lipid content took the longest time to develop into prepupae. The L3.5 treatment had the best results in terms of weight, but, also in this case, the high SEM of the L1.5 treatment did not allow obtaining a statistical evidence. In terms of development time, the L3.5 performed best when compared to the treatments with lower lipid levels. In Arthropoda, if the nutritional requirements are not met, in quality or/and quantity, the development time can vary[24]. In the present study, the treatment with a lipid content greater than or equal to 1.5% on DM performed the shortest development time. Therefore, a reduced quantity of lipids can determine an increase in larval stage duration. The prepupae weight and the L–P duration time recorded in the present study differ from the results obtained by Tomberlin et al.[33]. The L1 group, the least performing, had an average weight higher than the best treatment of Tomberlin et al.[33]. Time needed to reach prepupal stage was also shorter in this study when compared to that was found by Tomberlin et al.[33]. Since the nutrient composition in the two studies was similar, these differences can be attributed to different breeding methods rather than to the diets. In particular, in the study conducted by Tomberlin et al.[33], daily feeding was applied in opposition to the one-time feeding of the present trial.

Pupal stage. Similarly to the prepupa phase, the L1 treatment showed the lowest weight and longest development time. Lipid levels below 1.5% on DM, when compared to 4.5%, resulted in a smaller pupa size (as a logical consequence of the lower larval and prepupal weights). Since the metamorphosis of the adult occurs in the puparium, a small pupa generates proportionately equal adults. In the three isonitrogenous and isolipidic diets with increasing metabolizable energy (from 2174.5 to 3044.5 kcal/kg) formulated by Georgescu et al.[26], no difference in pupal weight was found. From a reproductive point of view, therefore, defining the optimal lipid level in the larval stage could allow for greater performance in the adult stage. Considering the development time, as stated in the previous sub-paragraph, the larval stage is the most important phase for the nutrient accumulation. For this reason, the longest development time of L1 at the prepupae stage affected, logically, the pupa's time to reach.

Adult stage. Since the flies lived in a box with no intraspecific interaction and with a reduced energy consumption due to the low possibility of movement, the experimental conditions of the adult phase are not comparable to the breeding ones. However, considering that all the experimental treatments were subjected to the same environmental effects, the results obtained are attributable to the diets. The experimental diets influenced the FLS, and data recorded during the trial are partially in agreement with those of Bellezza Oddon et al.[23], in which the diet with EE values close to 1% (CP14, CP16 and CP19) lived up to one day longer than L1 (8.43, 8.45, 9.36 days versus 8.05 day). Chia et al.[36] and Qomi et al.[37] observed the flies' longevity at different temperature, and at 30 °C the average lifespan lies within the 5–10 days range – period comparable to the FLS of the semi-purified diets. Since the major numerical WR was manifested by the group with the least longevity (L1.5) and there is no clear relationship trend between the two variables, it is possible to hypothesize that weight loss is not influenced by FLS. The FLW was greater in the dietary treatment with a lipid level above 3.5% on DM when compared to L1 and L1.5 groups. Although there is no statistical difference, on a numerical level it is observable that diets below 1.5% of EE had a reduced weight when compared to the others. The live weight parameter varies widely even if related with similar diets in term of nutritional composition. For example, Georgescu et al.[26] observed that the weight of flies derived from larvae fed on substrates with about 13.5% of CP and 3% of EE

on DM lies in a range between 0.028 and 0.039 g, thus being much lower than the ones recorded in the present study (range: 0.082–0.101 g). Since in literature there are few studies concerning nutrients intake effects on the flies, it is difficult to hypothesize an exclusive diet influence—but more probably an interaction between diet and genetic.

As reported by Bellezza Oddon et al.[23], the protein content in the diet does not influence the P–F duration time. On the contrary, the lipid percentage seems to have an effect on the intra-puparium development time. In particular, P–F time tended to get longer as the lipid level increased. The intra-puparial development can be influenced by several factor like temperature, pupation site and feed ingested during larval stage[34]. As regard the feed ingestion, the provision of inadequate feed can speed up this phase of BSF life cycle[34]. For this reason, it is possible to state that the L4.5% was a more complete diet since took longer P–F time when compared to diets with a lipid percentage below 2.5%. Few nutritional reserves combined with rapid development is a maximization of resources and increases, in case of unfavourable conditions, the probability of survival.

No differences were observed for the PW that was, logically, numerically lower in L1, the diet with the smaller flies, and higher in L3.5, in which the flies were bigger. Finally, the lipid percentage of the diet does not influence the ER, differently from what was assumed by Bellezza Oddon et al.[23]. Moreover, the sex ratio is not affected by the treatment, and in all the experimental diets the ratio was in favour of males as observed by Barragán Fonseca et al.[38] in a vegetable-based diet with 10% of CP and 5.7% of EE on DM.

All the adult parameters analysed in the current study were affected by the sex. Data about the effect of the sex on the pupae development are still very limited. In the present study, the P–F duration time was longest in females. In contrast, no significant differences between sex in the pupae development were noted by Tomberlin and Sheppard[39]. Since the female spends more time in larval stage, it is possible to speculate that the same condition may occur in pupal phase, in which the formation of female reproductive system requires the longest period[40]. As noted by Bellezza Oddon et al.[23], the fly sex had an effect on the life span, with the males living longer than females. Tomberlin et al.[41] observed that males lived 3.4 d or 3.5 d longer than females at 27 °C and 30 °C, without evident temperature effects. In the present study, male's lifespan was 1.6 d longer compared to females, and the difference between the two outcomes could be attributed to the nutrient accumulation during the larval stage and/or to genetics. The reduced lifespan of the females may be explained as a procreator mechanism. Since females did not display any multiple mating attempts during Jones et al.[42] observations, it is possible to hypothesize that males mate more times than females and, consequently, a longer life span can allow it to have more fecundation success. In some insect species, the female mating occurs only once in the course of life, as, for example, many mosquitoes appear to be monandrous[43]. In other cases, as for *Epiphyas postvittana* (Walker; *Lepidoptera*: *Tortricidae*), the multiple mating of females is generally attributed to a male dysfunction[44]. As known in literature, the fly and the puparium weight are sex-related: females have a greater weight than males and, logically, also the puparium size follows the same trend[41]. The sex-linkage between lifespan and weight can be explained as an adaptative system derived from sex-specific optimization of reproduction and survival trade-off[45]. In particular, the highest female weight may derive from the necessity to accumulate a consistent amount of nutrients in the larval stage to produce eggs[41]. On the contrary, males live longer but have the lowest weight in order to have the most time possible to fecundate. Despite the shorter lifespan of the female, the percentage weight loss of the fly was greater in females than in males. It is possible to assume that the higher energy accumulated in larval stage by the female for egg production is consumed also in male absence. Indeed, it was hypothesized that, if the flies do not have the opportunity to mate early after the emergence, the oocytes are reabsorbed in order to sustain other vital functions[39]. Therefore, the female reduced clutch size (oocytes) may be the cause of the increased weight loss compared to the male.

Conclusion

The determination of the macronutrients needs in the diets of the BSF represents one of the basis for the production maximization. As regards the lipids, in the whole larval stage and in prepupae/pupae phases, percentages lower than or equal to 1% of EE on the DM have a negative effect on growth (smallest size and longest development time). In the larvae earlier stages (up to 10 days), however, a lipid level equal to 4.5% allows obtaining better growth performance.

Based on this outcome, it is possible to hypothesize a greater lipid requirement during the first stages of growth, which tends to be less evident in the later larvae instars. The results obtained at the adult stage do not allow identifying an optimal diet at this level.

To the authors knowledge, the present study is the first one on the BSF lipid requirement assessment. Considering the reduced lipid content in the waste, low fat content levels has been tested. In addition, in order to maintain isoenergetic diets, only the range between 1.5 and 4.5% was evaluated. Since the results obtained are the inception of the optimal lipid level determination, further researches will be needed to evaluate the effect on BSF life history traits of percentage above 4.5% of EE on DM.

Materials and methods

Experimental diets. Five experimental diets with increasing percentage of lipids on DM, namely: 1% (L1), 1.5% (L1.5), 2,5% (L2.5), 3,5% (L3.5) and 4.5% (L4.5) were tested. The Gainesville diet (GA)[22], which is normally used in the experimental facility (Tetto Frati—Department of Agricultural, Forest and Food Sciences; University of Turin) for the colony maintenance, was used as environmental control diet to assess the non-anomalies occurrence during the experiment and the quality of the larval batch. Therefore, data collected from this diet were not used for the statistical analysis. To prepare the GA, corn seeds and alfalfa pellets were ground in powder with particles smaller than 2 mm using a grinding mill (CL/5 Fimar, Italy) and successively mixed with wheat bran according to Hogsette[46].

Ingredients	Casein	Cocoa butter	Wheat starch
DM	92.05	99.81	91.13
CP	92.61	0.32	2.56
EE	0.30	99.62	0.03
Ash	4.05	0.05	0.63
aNDFom	0.00	0.00	0.00
NSC[1]	2.94	0.00	71.24
GE, MJ/kg	23.52	39.82	17.43

Table 5. Chemical composition (g/100 g as is) and gross energy of the semi-purified ingredients used for the 5 experimental diets. DM: dry matter, CP crude protein, EE ether extract, aNDFom amylase neutral detergent fiber organic matter, NSC non-structural carbohydrates, GE gross energy. [a]Values are reported as mean of duplicate analyses. [1]Calculated as 100−[(100−DM) + CP + EE + Ash + aNDFom].

Items	L1	L1.5	L2.5	L3.5	L4.5	GA
Starch	833	827.5	822.5	817	812	-
Casein	160	160	160	160	160	-
Cocoa butter	7	12.5	17.5	23	28	-
Corn	-	-	-	-	-	200
Alpha-alpha	-	-	-	-	-	300
Wheat bran	-	-	-	-	-	500
Total	1000	1000	1000	1000	1000	1000
Chemical composition[a]						
DM	91.21	91.25	91.45	91.64	91.51	82.77
CP	17.36	17.39	17.51	17.31	17.47	15.28
EE	0.94	1.46	2.54	3.50	4.65	2.94
Ash	1.07	1.06	1.03	1.04	1.04	6.46
aNDFom	0.46	0.89	0.43	0.28	0.51	0.16
NSC[1]	80.17	79.20	78.49	77.87	76.33	75.16
GE	15.45	15.62	15.92	16.01	16.06	18.92

Table 6. Ingredients (g/kg, as is), chemical composition (g/100 g on DM) and gross energy (MJ/kg, on DM) of the 5 experimental substrates and the Gainesville diet. *Legend*: DM: dry matter, CP crude protein, EE ether extract, aNDFom amylase neutral detergent fiber organic matter, NSC non-structural carbohydrates, GE gross energy. [a]Values are reported as mean of duplicate analyses. [1]Calculated as 100 − (CP + EE + Ash + aNDFom).

The semi-purified ingredients (wheat starch, casein and cocoa butter; Table 5) were analysed in order to know their exact nutritional values to allow the formulation of the experimental diets. All the raw ingredients were measured and mixed with warm tap water (28 °C) to achieve 70% moisture content in the diets. After the preparation, a sample of each experimental diet and GA (150 g) were chemically analysed (Table 6).

Growth trial. One-day-old larvae were provided by a European insect producer (Hermetia Baruth GmbH Baruth/Mark, Germany), and were reared for 5 days on GA in a climatic chamber under controlled environmental conditions (T°: 28 ± 0.5 °C; RH: 70 ± 5%; 0:24 L:D), using plastic boxes (19 cm × 13 cm × 6 cm) cover by a lid with nylon grid to allow air exchange.

To increase homogeneity in the sample, 6-days-old larvae were sieved with a mesh diameter of 0.8 mm, and only the larvae which passed through the sieve were used. To obtain 6 replicates for each diet, 20 groups of 5 larvae were weighed (Kern & Sohn GmbH; Balingen, Germany; d = 0.001) and then, if the standard deviation of the larvae weight was less than 0.02, they were pooled together (100 larvae/replicate).

Each replicate consisted of a plastic box (14 × 14 × 7 cm) filled with 180 g of substrate (1.8 g/larvae) and covered with a nylon grid to allow air exchange. Larvae were directly distributed on the substrate after the acclimation of the latter in the climatic chamber, in order to avoid a thermal shock for the formers. The plastic boxes were kept in the same climatic chamber where the 1-day-old larvae were reared.

Larval stage. Each replica was observed daily to monitor the evolution of the substrate, and replicates were randomly redistributed to avoid bias from temperature being slightly higher at the top of the shelves in the climatic chamber. Every 4 days from the first day of the trial, 30 larvae were randomly taken from each replica by carefully avoiding the substrate removal. The BSFL were washed, gently dried with a paper tissue, and weighed. As the measure was not destructive, BSFL were put back in their box. This operation was repeated up until a

replica reached 40% of the BSFL turning into prepupae, the moment in which the larval stage was considered ended in accordance to Tomberlin et al.[33]. To know exactly when a replica reached 40% of prepupae, all the replicates were daily checked after the first prepupa was observed. At the end of larval stage, the total number of larvae and prepupae was assessed for the survival calculation and the time interval from larva to prepupa was recorded (L-Pp, days). For the evaluation of the prepupae growth performance, 30 prepupae were randomly taken, washed, gently dried with paper tissue, weighed (Kern & Sohn GmbH; Balingen, Germany; d = 0.001), and put back in their box with the rest of the larvae to let them reach the pupal stage.

Chemical analysis. The semi-purified ingredients, the diets and the larvae were stored at -20 °C. The larvae were ground (Retsch, GM 200) as frozen and freeze-dried. For the proximate composition analysis, the dry matter (DM; AOAC #934.01), the crude protein (CP; AOAC #984.13; conversion factor for ingredients and diets N × 6.25, for larvae N × 4.67[47]) and the ash (AOAC #942.05) were determined by the International AOAC[48], the ether extract (EE; AOAC #2003.05) by the International AOAC[49], and the amylase neutral detergent fiber and organic matter (aNDFom) by Mertens[50]. The non-structural carbohydrates (NSC) were calculated as difference with the other nutrients. The gross energy (GE) was analysed using an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany).

Pupal and adult stages. To collect 23 pupae (characterized by their rigidity and immobility) per replicate for the determination of pupa and adult parameters, each replica was daily checked. When a pupa was found, it was cleaned without washing it, weighed, and then put in a transparent plastic "emergence box" (circular, 8 cm of diameter, 4 cm height; 1 pupa/box) with a perforated transparent plastic lid. The time interval from larva to pupa was also recorded (L–P, days).

Emergence box weight (EBW, g) was recorded before inserting the pupa. The emergence boxes were kept in the same climatic chamber used for rearing the larvae and were daily checked for the presence of flies. Once a fly emerged, the emergence box (with the puparium and the fly) was weighted (EBW_PF, g) to calculate the fly live weight (FLW, g) and the sex of the fly was assessed. The date of the emergence was recorded to evaluate the duration of the fly's development (pupa-fly [P–F]). The box was put back in the climatic chamber until the death of the fly. Once the fly died, the emergence box was opened, the puparium (PW, g) and the dead fly (DFW, g) were weighed separately, and the sex of the fly checked a second time for accuracy. The DFW was used to calculate the weight reduction of the flies (WR, %). The fly death date was recorded in order to calculate the lifespan of the fly (FLS, days).

The formulas to calculate the parameters which were not directly measured are:

$$\text{Fly Live Weight (FLW, g)}\ FLW = EBW_PF - EBW - PW$$

$$\text{Weight Reduction (WR, \%)}\ \text{WR} = \text{FLW} - \left(\frac{DFW}{FLW} \times 100\right)$$

Emergence rate and sex ratio. To assess the overall emergence rate (ER) and sex ratio (SR; female-to-male ratio), all the remaining larvae/prepupae of each replica were left untouched in the climatic chamber until fly emergence. Once all flies' dead, the two parameters were calculated.

$$ER = \frac{n^\circ emerged\ flies\ \times 100}{n^\circ pupae\ collected\ per\ treatment}$$

Larvae descriptive nutritional composition. Simultaneously to the growth trial, a larger scale experiment was set up with the aim of collecting the larvae samples for the chemical composition. A total of 2 replicates per each dietary treatment were performed in a 23 × 30 × 9 cm box with a lid with perforated nylon to allow the air circulation. Each replica consisted of 1500 6-day-old larvae sieved with a 0.8 mm mesh and fed 2700 g of experimental diet (1.8 g/larva). The number of larvae was estimated by sampling (3 samples taken, with a coefficient of variation among the samples < 10%). The BSFL were left feeding on the experimental diets under controlled environmental conditions (T°: 28 ± 0.5 °C; RH: 70 ± 5%; 0:24 L:D). The boxes were daily observed and, when the first prepupa was detected, the larvae were collected, washed, gently dried with paper tissue, and inactivated at −80 °C. Larvae samples were processed and analysed using the method listed in the chemical analysis paragraph.

Statistical analysis. Since the GA was considered only as environmental control, it was excluded from the statistical analysis (data reported in the Supplementary Information). Data were analysed using the IBM SPSS Statistics software (V20.0.0.). The statistical unit for the parameters recorded during the larval, prepupal, pupal and adult stages was the individual, with the exception of ER and SR, calculated as percentage or ratio of the total, in which it was the replicate. Shapiro–Wilk test was performed in order to evaluate the normality of the residuals, while the assumption of equal variances was assessed by Levene's homogeneity of variance test. A generalized linear mixed model (GLMM) with a gamma probability distribution (nonlinear link function [log]) was fitted for the larvae weight and adult parameters, with two fixed factors being considered (diet/time for the larvae and diet/sex for the flies, plus their interaction). If the interaction was not significant, a likelihood-ratio was carried out and, when necessary, a model simplification by removing it was applied. In order to account the

repeated measurements during the time, replicates were included in the model as a random effect. The interactions between the levels of the fixed factors were evaluated by means of pairwise contrasts.

Since in prepupal and pupal stages the replicate was considered as random effect, an analogous GLMM was applied. The ER and SR were, instead, analysed by one-way ANOVA test (post-hoc test: Tukey). The results were expressed as mean and pooled standard error of the mean (SEM). The level of significance considered was < 0.05.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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References

1. Hawkey, K. J., Lopez-Viso, C., Brameld, J. M., Parr, T. & Salter, A. M. Insects: a potential source of protein and other nutrients for feed and food. *Annu. Rev. Anim. Biosci.* **9**, 333–354 (2020).
2. van Huis, A. Insects as food and feed, a new emerging agricultural sector: a review. *J. Insect. Food Feed* **6**, 27–44 (2020).
3. Ravi, H. K. *et al.* Larvae mediated valorization of industrial, agriculture and food wastes: biorefinery concept through bioconversion, processes, procedures, and products. *Process* **8**, 857 (2020).
4. Fowles, T.M., Nansen, C., Insect-based bioconversion: value from food waste. Food Waste Management (eds Närvänen E., Mesiranta N., Mattila M., Heikkinen A) 321–346 (Macmillan, New York, 2020).
5. Kaya, C. *et al.* Global population genetic structure and demographic trajectories of the black soldier fly, *Hermetia illucens*. *BMC Biol.* **19**, 94. https://doi.org/10.1186/s12915-021-01029-w (2021).
6. Zhan, S. *et al.* Genomic landscape and genetic manipulation of the black soldier fly Hermetia illucens, a natural waste recycler. *Cell Res.* **30**, 50–60 (2019).
7. Gasco, L., Biancarosa, I. & Liland, N. S. From waste to feed: a review of recent knowledge on insects as producers of protein and fat for animal feeds. *Curr. Opin. Green Sustain. Chem.* **23**, 67–79 (2020).
8. Kim, C. H. *et al.* Use of black soldier fly larvae for food waste treatment and energy production in asian countries: a review. *Processes* **9**, 161. https://doi.org/10.3390/pr9010161 (2020).
9. Norgren, R., Jonsson, A. & Björkqvist, O. Original article: fermented pulp and paper bio-sludge as feed for black soldier fly larvae. *Biomass Conv. Bioref.* **10**, 1007. https://doi.org/10.1007/s13399-021-01564-0 (2021).
10. Ojha, S., Bußler, S. & Schlüter, O. Food waste valorisation and circular economy concepts in insect production and processing. *J. Waste. Manag.* **118**, 600–609 (2020).
11. IPIFF. The European insect sector today: challenges, opportunities and regulatory landscape. IPIFF vision paper on the future of the insect sector towards 2030. https://ipiff.org/wp-content/uploads/2019/12/2019IPIFF_VisionPaper_updated.pdf (2019).
12. Aarts, K. W. P. How to develop insect-based ingredients for feed and food? A company's perspective. *J. Insect. Food Feed* **6**(1), 67–68 (2020).
13. Cadinu, L. A., Barra, P., Torre, F., Delogu, F. & Madau, F. A. Insect rearing: potential, challenges, and circularity. *Sustainability* **12**, 4567. https://doi.org/10.3390/su12114567 (2020).
14. Mancuso, T., Pippinato, L. & Gasco, L. The european insects sector and its role in the provision of green proteins in feed supply. *Calitatea* **20**, 374–381 (2019).
15. Scott, H. M., Moellek, M. W. & Hinnees, S. W. Studies with purified diets. *Poult. Sci. J.* **36**, 1000–1002 (1957).
16. Oltjen, R. R., Sirny, R. J. & Tillman, A. D. Purified diet studies with sheep. *J. Anim. Sci.* **21**, 277–283 (1962).
17. Kim, K. I., Kayes, T. B. & Amundson, C. H. Purified diet development and re-evaluation of the dietary protein requirement of fingerling rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **96**, 57–67 (1991).
18. Cammack, J.A., Tomberlin, J.K. The impact of diet protein and carbohydrate on select life-history traits of the black soldier fly *Hermetia illucens* (L.) (*Diptera: Stratiomyidae*). Insects **8**, 56; doi:https://doi.org/10.3390/insects8020056 (2017).
19. Barragan-Fonseca, K. B., Gort, G., Dicke, M. & van Loon, J. J. A. Effects of dietary protein and carbohydrate on life-history traits and body protein and fat contents of the black soldier fly *Hermetia illucens*. *Physiol. Entomol.* **44**, 148–159 (2019).
20. Aguila, J. R., Hoshizaki, D. K. & Gibbs, A. G. Contribution of larval nutrition to adult reproduction in *Drosophila melanogaster*. *J. Exp. Biol.* **216**, 399–406 (2013).
21. DiAngelo, J. R. & Birnbaum, M. J. Regulation of fat cell mass by insulin in *Drosophila melanogaster*. *Mol. Cell. Biol.* **29**, 6341–6352 (2009).
22. Spindles, K. D. *et al.* Ecdysteroid hormone action. *Cell. Mol. Life Sci.* **66**, 3837–3850 (2009).
23. Bellezza Oddon, S., Biasato, I. & Gasco, L. Isoenergetic-practical and semi-purified diets for protein requirement determination in *Hermetia illucens* larvae: consequences on life history traits. *J. Animal Sci. Biotechnol.* **13**, 17. https://doi.org/10.1186/s40104-021-00659-y (2022).
24. Spindles, K. D. *et al.* Ecdysteroid hormone action. *Cell. Mol. Life. Sci.* **66**, 3837–3850 (2009).
25. Nguyen, T. T. X., Tomberlin, J. K. & Vanlaerhoven, S. Influence of resources on *Hermetia illucens* (Diptera: *Stratiomyidae*) larval development. *J. Med. Entomol.* **50**, 898–906 (2013).
26. Georgescu, B., Struți, D., Păpuc, T., Cighi, V. & Boaru, A. Effect of the energy content of diets on the development and quality of the fat reserves of larvae and reproduction of adults of the black soldier fly, *Hermetia illucens* (Diptera: *Stratiomyidae*). *Eur. J. Entomol.* **118**, 297–306 (2021).
27. Cohn, Z., Latty, T. & Abbas, A. Understanding dietary carbohydrates in black soldier fly larvae treatment of organic waste in the circular economy. *J. Waste Manag.* **137**, 9–19 (2022).
28. Miranda, C.D., Cammark, J.A., Tomberlin, J.K. Life-history traits of the black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae), reared on three manure types. Animals **9**(5), 281. https://doi.org/10.3390/ani9050281 (2019).
29. Jones, B. M. & Tomberlin, J. K. Impact of larval competition on life-history traits of the black soldier fly (*Diptera: Stratiomyidae*). *Ann. Entomol. Soc. Am.* **112**, 505–510 (2019).
30. Barragan-Fonseca, K.B., Dicke, M., van Loon, J.J.A. Nutritional value of the black soldier fly (*Hermetia illucens* L.) and its suitability as animal feed: a review. J. Insects Food Feed **3**, 105–120 (2017).
31. Pearincott, J. V. Changes in the lipid content during growth and metamorphosis of the house fly, *Musca domestica* Linnaeus. *J. Cell. Physiol.* **55**, 167–174 (1960).
32. Meneguz, M. *et al.* Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (*Hermetia illucens*) larvae. *J. Sci. Food Agr.* **98**, 5776–5784 (2018).
33. Tomberlin, J. K., Sheppard, D. C. & Joyce, J. A. Selected life-history traits of black soldier flies (Diptera: *Stratiomyidae*) reared on three artificial diets. *Ann. Entomol. Soc. Am.* **95**, 379–386 (2002).

34. Li, L., Wang, Y., Wang, J. Intra-puparial development and age estimation of forensically important *Hermetia illucens* (L.). J. Asia Pac. Entomol. **19,** 233–237 (2016).
35. Gold, M. *et al.* Biowaste treatment with black soldier fly larvae: increasing performance through the formulation of biowastes based on protein and carbohydrates. *J. Waste Manag.* **102**, 319–329 (2020).
36. Chia, S. *et al.* Effects of waste stream combinations from brewing industry on performance of Black Soldier Fly, *Hermetia illucens* (*Diptera: Stratiomyidae*). *Zool. Sci.* https://doi.org/10.7717/peerj.5885 (2018).
37. Qomi, M. F., Danaeefard, M., Farhang, A., Hosseini, P. & Arast, Y. Effect of temperature on the breeding black soldier fly larvae in vitro for basic health-oriented research. *Arch. Hyg. Sci.* **10**, 67–74 (2021).
38. Barragán Fonseca, K. B., Dicke, M. & van Loon, J. J. A. Influence of larval density and dietary nutrient concentration on performance, body protein, and fat contents of black soldier fly larvae (*Hermetia illucens*). *Entomol. Exp. Appl.* **166**, 761–770 (2018).
39. Tomberlin, J. K. & Sheppard, D. C. Factors influencing mating and oviposition of black soldier flies (Diptera: Stratiomyidae) in a colony. *Entomol. Sci.* **37**, 345–352 (2002).
40. Hoc, B., Noël, G., Carpentier, J., Francis, F. & Megidoet, R. C. Optimization of black soldier fly (*Hermetia illucens*) artificial reproduction. *PLoS ONE* https://doi.org/10.1371/journal.pone.0216160 (2019).
41. Tomberlin, J. K., Adler, P. H. & Myers, H. M. Development of the black soldier fly *(Diptera: Stratiomyidae*) in relation to temperature. *Environ. Entomol.* **38**, 930–934 (2009).
42. Jones, B.M., Tomberlin, J.K. Effects of adult body size on mating success of the black soldier fly, *Hermetia illucens* (L.) (Diptera: *Stratiomyidae*). J. Insect. Food Feed **7,** 5–20 (2021).
43. South, S. H. & Arnqvist, G. Evidence of monandry in a mosquito (*Sabethes cyaneus*) with elaborate ornaments in both sexes. *J. Insect. Behav.* **21**, 451–459 (2008).
44. Foster, S. P. & Ayers, R. H. Multiple mating and its effects in the light brown apple moth, *Epiphyas postvittana* (Walker). *J. Insect. Physiol.* **42**, 657–667 (1995).
45. Maklakov, A. A. & Lummaa, V. Evolution of sex differences in lifespan and aging: causes and constraints. *BioEssays* **35**, 717–724 (2013).
46. Hogsette, J. A. New diets for production of house flies and stable flies (*Diptera: Muscidae*) in the laboratory. *J. Econ. Entomol.* **85**, 2291–2294 (1992).
47. Janssen, R. H., Vincken, J. P., van den Broek, L. A., Foglianom, V. & Lakemond, C. M. Nitrogen-to-Protein Conversion Factors for Three Edible Insects: *Tenebrio molitor*, *Alphitobius diaperinus*, and *Hermetia illucens*. *J. Agric. Food Chem.* **22**, 2275–2278 (2017).
48. AOAC International. Official methods of analysis of AOAC International. 17th ed. Gaithersburg: Association of Official Analytical Chemists (2000).
49. AOAC International. Official methods of analysis of AOAC International. 17th ed.; 2nd revision. Gaithersburg Association of Official Analytical Chemists (2003).
50. Mertens, D. R. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. *J. AOAC Int.* **85**, 1217–1239 (2002).

Author contributions

S.B.O., I.B. and L.G. conceived and designed the experiment. S.B.O., I.B., A.R. and L.G. performed the trial and collected the experimental data. S.B.O. and I.B. analysed the data. S.B.O. carried out the chemical analyses. S.B.O. and A.R. wrote the first draft of the manuscript. All the authors critically reviewed the manuscript for its intellectual content and gave their approval for the final version to be published.

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Competing interests

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Correspondence and requests for materials should be addressed to I.B.

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Dietary inclusion of a partially defatted black soldier fly (Hermetia illucens) larva meal in low fishmeal-based diets for rainbow trout (Oncorhynchus mykiss)

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Dietary inclusion of a partially defatted black soldier fly (*Hermetia illucens*) larva meal in low fishmeal-based diets for rainbow trout (*Oncorhynchus mykiss*)

Christian Caimi[1], Ilaria Biasato[1], Giulia Chemello[1], Sara Bellezza Oddon[1], Carola Lussiana[1], Vanda Maria Malfatto[1], Maria Teresa Capucchio[2,3], Elena Colombino[2], Achille Schiavone[2], Francesco Gai[3], Angela Trocino[4], Alberto Brugiapaglia[1], Manuela Renna[2*] and Laura Gasco[1]

Abstract

Background: Recent investigations highlighted that *Hermetia illucens* (BSF) larva meal can be a valuable alternative protein source for aquafeed production. In this study, in substitution of fishmeal, we used increasing inclusion levels of a partially defatted BSF larva meal in low fishmeal-based diets for rainbow trout (*Oncorhynchus mykiss* Walbaum) and we evaluated the related implications in terms of growth performance, physical characteristics, proximate and fatty acid (FA) compositions of the fillets, gut and liver histology, and diet digestibility. In a 131-day trial, 576 fish (100.1 ± 9.29 g) were randomly allotted to 24 tanks (24 fish/tank, 4 replicates/treatment). Six experimental diets were produced to have partial replacement of fishmeal (0, 10%, 20%, 30%, 40% and 50%) by increasing levels of BSF meal (0% [BSF0], 3% [BSF3], 6% [BSF6], 9% [BSF9], 12% [BSF12] and 15% [BSF15] on as fed basis, respectively).

Results: No differences were observed among the treatments for all the considered growth performance parameters. The viscero and hepato-somatic indexes showed significant differences among the treatments, with the highest values observed in the BSF15 group. No differences were recorded in terms of fillet's physical characteristics, dry matter (DM), crude protein (CP) and ether extract (EE) contents. Total saturated and monounsaturated FA increased, while polyunsaturated FA (particularly n-3 FA) decreased while increasing the HI meal inclusion in the diet. Histopathology of liver and gut was not affected, whereas, in posterior gut, villi were higher in BSF6 and BSF9 compared to BSF3 fish. The apparent digestibility of DM, CP, EE and gross energy did not vary among the treatments.

Conclusions: These results suggest that a partially defatted BSF meal could be included up to 15% in low fishmeal-based diets for rainbow trout with no adverse effects on growth performance, fillet's physical characteristics, gut and liver health, and diet digestibility. On the contrary, the fillet FA composition worsened while increasing the level of BSF meal in the diet.

Keywords: Apparent digestibility coefficient, Chemical and physical characteristics of fillets, Fatty acid profile, Fishmeal substitution, *Hermetia illucens* meal, Histopathology, Performance

* Correspondence: manuela.renna@unito.it
[2]Department of Veterinary Sciences, University of Turin, Largo P. Braccini 2, 10095 Grugliasco, TO, Italy
Full list of author information is available at the end of the article

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Background

In the next few years, there will be an increase in the global demand of protein because of the constant increase in the world population [1]. Aquaculture has the fastest growing in the food production, with an average annual rate above 5.5% per year [2] and, for this reason, is considered as one of the livestock sectors able to support the global demand of animal products [3]. The growing in fish production leads to an increase in the demand of aquaculture feeds. For many years, fishmeal (FM) has been the preferred protein source for the production of aquafeeds due to its valuable content of protein and fatty acids (FA), amino acid profile, high digestibility and palatability [4]. However, the constant increase of the demand of aquaculture feeds has led to a rapid growth in the FM price and to a negative impact on the marine ecosystem [5]. In order to maintain the correct percentage of protein to meet the nutritional requirements of fish, in the last 20 years FM has been partially replaced with alternative raw materials, such as vegetable protein sources and processed animal proteins (PAPs), leading to a reduction in the dietary FM inclusion in the feeds.

Recently, in order to replace FM, the attention has been focused on the use of insect-derived PAPs. Insects can be used to produce high quality ingredients, rich in protein and fat, starting from waste biomass and with low environmental impact [6, 7]. One of the insect species with the highest potential to be used in fish feeds is the black soldier fly (*Hermetia illucens* L.) (BSF). The larvae of this fly can be reared on low value organic waste, with low water demand and generating low greenhouse gas emissions [8]. Generally, the meals obtained from BSF show a protein content ranging from 37% to 63%, and a fat content from 7% to 39% on a dry matter (DM) basis [9, 10].

Despite the nutritional value of *BSF meal,* its successful inclusion level in aquafeeds depends also on their effect on gut health and liver integrity, which are fundamental for nutrient digestion and absorption and thus for growth performances [11]. For this reason, gut histomorphometry and liver histopathology are usually taken into account when alternative ingredients are investigated in animal nutrition [12]. Nutritional studies on the total and partial substitution of FM with BSF meal have been previously conducted in rainbow trout (*Oncorhynchus mykiss* Walbaum). A recent study by Cardinaletti et al. [12] showed that in a control diet containing 42% of FM, the 50% of the FM could be replaced including a dietary inclusion up to 21% of a full-fat BSF meal without negative effects on the growth performance of rainbow trout. However, a decrease in villus height and an increase in liver lipid accumulation was observed in trout fed BSF meal. Compared to a control diet with a FM dietary inclusion of 60%, Renna et al. [13] showed that an inclusion up to 40% of partially defatted BSF meal could replace up to 50% of FM without any adverse effects on growth performance, gut and liver histomorphometry. On the contrary, studies by Dumas et al. [14] and St-Hilaire et al. [15], using a defatted BSF meal (up to 26.4% of inclusion) and a full-fat BSF meal (up to 29.8% of inclusion), respectively, showed a worsening of the growth performance of trout at increasing BSF inclusion levels. The growth performance and the gut and liver histopathology reported in these studies showed contradictory results probably due to several factors, such as the nutritional composition and inclusion level of the insect meal, and fish size.

As we can see in literature, most of the studies performed in fish nutrition replacing FM with BSF meal have been conducted using control diets with high levels of FM, usually higher than 30% [13, 16–20]. However, due to the current price of FM, only 10–20% of FM is currently included in commercial diets [2, 21].

Therefore, the aim of this study was to determine the potential of six inclusion levels of a partially defatted BSF larva meal as a partial replacer of FM in low FM-based diet, on growth performance, somatic indexes, fillet's physical characteristics, proximate composition and FA profile, histopathological investigation of gut and liver, and digestibility in rainbow trout.

Methods

The experimental protocol was designed according to the guidelines of the current European Directive on the protection of animals used for scientific purposes (2010/63/EU) and approved by the Ethical Committee of the University of Turin (Italy) (protocol n° 143811). The trial was carried out at the Experimental Facility of the Department of Agricultural, Forest and Food Sciences (DISAFA) of the University of Turin (Italy).

Experimental diets

Six experimental diets were formulated to be isonitrogenous (crude protein – CP: about 45.8 g/100 g as fed), isolipidic (ether extract – EE: about 15.2 g/100 g as fed), and isoenergetic (gross energy – GE: about 22.6 MJ/kg as fed). The six diets were obtained including, as fed basis, increasing levels of a partially defatted BSF larva meal – 0, 3%, 6%, 9%, 12% and 15% – corresponding to a substitution of 0% (BSF0), 10% (BSF3), 20% (BSF6), 30% (BSF9), 40% (BSF12) and 50% (BSF15) of FM. The BSF larva meal used in this study was provided by MUTATEC (Caumont-sur-Durance, France). The larvae had been raised on plant by-products and partially defatted using a mechanical process. Unfortunately, no other information was given by the producer about either the rearing substrate or the processing methodologies, as

this information is considered confidential. The experimental diets were prepared at the Experimental Facility of DISAFA. All the ground ingredients and oils were individually weighed (KERN PLE-N v.2.2; KERN & Sohn GmbH, Balingen-Frommern, Germany; d: 0.01) and mixed with a blender (Brevetti S.A.G.A., Milano, Italy). To facilitate the pelleting process, an amount of 250 to 500 mL/kg of water was added to the mixture. The pelletizing was performed using a meat grinder (LABOR 32; Rheninghaus Factory, San Mauro Torinese, Italy). The pellets (3.0 mm) were subsequently dried (50 °C for 48 h) and stored in black bags at − 20 °C until used. The ingredients of the experimental diets are reported in Table 1.

Chemical analyses of BSF meal and experimental diets

The proximate composition and the energy level of the BSF meal and experimental diets were measured in duplicate at the DISAFA laboratories. Feed samples were ground using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland) and analysed for DM (AOAC #934.01), CP (AOAC #984.13) and ash (AOAC #942.05) contents according to AOAC International [22]; EE (AOAC #2003.05) was analysed according to AOAC International [23]. The GE content was determined using an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany). The chitin content of BSF meal was estimated according to Finke [24] by correction considering the AA content of the acid detergent fiber (ADF) fraction and assuming the remainder of the ADF fraction is chitin. Due to the presence of vegetable raw material in the diets, the method proposed by Finke [24] cannot be applied to calculate the chitin content of the diets. For this reason, the amount of chitin of the experimental diets was estimated based on the chitin content of the BSF meal and its inclusion level in the diets.

The AA determination of BSF larva meal and experimental diets was performed according to the method described in De Marco et al. [25]. After a 22-h hydrolysis step in 6 mol/L HCl at 112 °C under a nitrogen atmosphere, the AA content in hydrolysate was determined by means of HPLC after postcolumn derivatization. Performic acid oxidation occurred prior to acid hydrolysis for methionine and cysteine. Tryptophan was not determined. The AA composition is shown in Table 2.

A combined direct trans-esterification and solid-phase extraction was carried out for the determination of the fatty acid profile of the BSF meal and experimental diets,

Table 1 Ingredients and proximate composition of the experimental diets and BSF larva meal

	BSF	BSF0	BSF3	BSF6	BSF9	BSF12	BSF15
Ingredients, %							
Fishmeal[a]		20.0	18.0	16.0	14.0	12.0	10.0
Hermetia illucens larva meal[b]		–	3.0	6.0	9.0	12.0	15.0
Wheat gluten		13.0	13.0	13.0	13.0	13.0	13.0
Soybean meal		20.0	20.0	20.0	20.0	20.0	20.0
Swine haemoglobin		9.2	9.0	8.8	8.6	8.4	8.2
Wheat starch		23.4	22.6	21.8	21.0	20.2	19.4
Fish oil		7.0	7.0	7.0	7.0	7.0	7.0
Soybean oil		7.0	7.0	7.0	7.0	7.0	7.0
Minerals[c]		0.25	0.25	0.25	0.25	0.25	0.25
Vitamins[d]		0.20	0.20	0.20	0.20	0.20	0.20
Chemical composition [e]							
Dry matter, g/100 g	94.0	97.2	97.2	96.9	96.8	96.9	96.6
Ash, g/100 g as fed	10.2	5.8	5.9	5.8	5.7	5.5	5.5
Crude protein, g/100 g as fed	56.9	45.6	46.1	45.6	46.0	45.7	46.1
Ether extract, g/100 g as fed	7.0	14.9	15.9	15.8	15.7	14.6	14.3
Gross energy, MJ/kg as fed	20.2	22.4	22.7	22.6	22.7	22.7	22.6
Chitin, g/100 g	6.3	–	0.18	0.37	0.56	0.75	0.93

Abbreviations: BSF Hermetia illucens. [a] Purchased from Skretting Italia S.p.A. (Località Vignetto, 17-37060 Mozzecane VR, Italy). Proximate composition (g/100 g, as fed): DM 91.0, CP 67.6, EE 8.3, Ash 16.4. [b] Provided by MUTATEC (Caumont-sur-Durance, France). [c] Mineral mixture (g/kg or mg/kg diet): dicalcium phosphate, 500 g; calcium carbonate, 215 g; sodium salt, 40 g; potassium chloride, 90 g; magnesium chloride, 124 g; magnesium carbonate, 124 g; iron sulphate, 20 g; zinc sulphate, 4 g; copper sulphate, 3 g; potassium iodide, 4 mg; cobalt sulphate, 20 mg; manganese sulphate, 3 g; sodium fluoride, 1 g (purchased from Granda Zootecnici S.r.l., Cuneo, Italy). [d] Vitamin mixture (IU/kg or mg/kg diet): *DL*-α-tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15,000 IU; *DL*-cholecalciferol, 3000 IU; Stay C Roche (vitamin C), 90 mg; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; vitamin B_{12}, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg (purchased from Granda Zootecnici S.r.l., Cuneo, Italy). [e] Values are reported as mean of duplicate analyses

Table 2 Amino acid (AA) concentration (g/100 g of protein) of BSF meal and experimental diets

	BSF	BSF0	BSF3	BSF6	BSF9	BSF12	BSF15
Essential AA							
Arginine	3.9	5.3	5.2	5.2	5.1	5.0	5.0
Histidine	2.2	2.8	2.8	2.8	2.8	2.7	2.9
Isoleucine	3.3	3.5	3.4	3.4	3.3	3.3	3.3
Leucine	5.2	7.7	7.5	7.5	7.3	7.2	7.1
Lysine	3.8	5.9	5.6	5.5	5.3	5.2	5.0
Methionine	2.6	2.5	2.5	2.5	2.5	2.5	2.6
Cysteine	1.3	1.3	1.2	1.1	1.1	1.0	1.3
Phenylalanine	3.0	4.6	4.6	4.5	4.5	4.4	4.3
Tyrosine	4.8	3.0	3.0	3.1	3.1	3.2	3.2
Threonine	3.1	7.6	7.6	7.6	7.6	7.6	7.6
Valine	4.9	3.5	3.5	3.4	3.4	3.3	3.3
Non-essential AA							
Alanine	6.2	4.8	4.8	4.8	4.8	4.8	4.8
Aspartic acid	6.7	8.1	7.9	7.9	7.7	7.7	7.6
Glycine	4.2	2.7	2.8	2.9	3.0	3.1	3.2
Glutamic acid	8.8	16.0	15.9	16.1	16.1	16.3	16.2
Proline	5.5	8.5	8.2	8.1	7.8	7.6	7.4
Serine	3.7	3.7	3.6	3.7	3.6	3.6	3.6

Abbreviations: BSF Hermetia illucens

using eptadecanoic acid as internal standard, as reported in Dabbou et al. [26]. Fatty acid methyl esters (FAME) were separated, identified and quantified as reported in Dabbou et al. [27]. The results are expressed as mg/100 g DM and are reported in Table 3.

All the chemical analyses of feed were performed in duplicate.

Fish and rearing conditions

A 131-day growth trial was carried out with rainbow trout purchased from a private fish hatchery ("Troticoltura Bassignana", Cuneo, Italy). An acclimatization period of two weeks was provided during which the fish were fed a commercial diet (42 g/100 g as fed of CP; 22 g/100 g as fed of EE; Skretting Italia Spa, Mozzecane (VR), Italy). Then a total of 576 fish were lightly anesthetised (60 mg/L MS-222 - PHARMAQ Ltd., Fordingbridge, Hampshire, UK), individually weighed (mean individual initial body weight – iIBW: 100.1 ± 9.29 g; KERN PLE-N v.2.2; KERN & Sohn GmbH, Balingen-Frommern, Germany; d: 0.01) and randomly divided into 24 fiberglass 200-L tanks (four replicate tanks per diet, twenty-four fish per tank). Artesian well water (constant temperature of 13 ± 1 °C) was supplied in flow-through open system with each tank having a water inflow of 8 L/min. Dissolved oxygen was measured every week and ranged between 7.6 and 8.7 mg/L. Feed was distributed by hand twice a day, six days per week (number of feeding days = 121). The fish were fed 1.4% of the tank biomass. Feed intake was checked at each administration and feed administration was stopped as soon as the fish stopped eating. In order to update the daily quantity of feed, the fish were weighed in bulk every 14 days. Mortality was checked every day.

Growth performance

At the end of the trial, after 24 h of fasting, all the fish were lightly anesthetised and individually weighed. The following performance indexes were calculated:

- Mortality (%) = (number of dead fish / initial number of fish) × 100
- Individual weight gain (iWG, g) = iFBW (average individual final body weight, g) – iIBW (average individual initial body weight, g)
- Specific growth rate (SGR, %/d) = [(lnFBW – lnIBW) / number of feeding days] × 100
- Feed conversion ratio (FCR) = total feed supplied (g, DM) / WG (g)
- Protein efficiency ratio (PER) = WG (g) / total protein fed (g DM).

Individual initial and final body weight were used to calculate the iWG while SGR, FCR and PER were calculated per tank.

Table 3 Fatty acid profile (mg/100 g DM) of BSF larva meal and experimental diets

	BSF	BSF0	BSF3	BSF6	BSF9	BSF12	BSF15
C10:0	67.8	43.8	60.3	73.4	91.0	49.7	63.2
C12:0	1898.7	6.94	64.7	101.1	177.9	228.4	291.4
C14:0	483.0	413.6	436.2	446.7	465.6	450.9	475.8
C15 *iso*	4.51	13.2	14.3	13.5	13.3	13.4	13.4
C15 *aiso*	5.74	4.62	4.63	4.54	4.12	4.24	4.34
C14:1 *c*-9 + C15:0	27.0	39.0	41.3	41.2	40.8	39.0	41.1
C16 *iso*	1.90	1.50	3.19	1.92	2.34	2.16	2.10
C16:0	1111.4	1901.7	1980.2	2072.9	2058.6	1966.5	2060.0
C17 *iso*	2.83	37.1	40.8	31.5	29.4	29.1	30.0
C17 *aiso*	29.8	11.5	18.6	7.66	4.75	5.85	6.20
C16:1 *c*-9	174.5	488.8	504.4	365.8	281.3	287.0	316.5
C17:1 *c*-9	8.16	14.9	18.5	10.3	8.06	8.38	9.06
C18:0	246.8	548.9	562.0	553.9	537.9	513.9	538.7
C18:1 *t*	14.5	11.0	11.0	9.43	9.24	9.13	10.9
C18:1 *c*-9	1216.0	2078.2	2171.7	1658.9	1278.1	1332.4	1530.6
C18:1 *c*-11	27.8	273.8	281.1	209.8	161.8	162.4	179.4
C18:1 *c*-12	0.32	6.22	3.49	1.25	1.43	1.00	1.16
C18:1 *c*-14 + *t*-16	4.42	11.41	8.85	5.38	4.79	4.16	5.01
C18:2 n-6	403.4	2381.2	2486.6	533.1	398.4	387.7	418.5
C18:3 n-3	26.0	241.3	244.8	30.4	24.3	22.7	21.7
C18:3 n-6	1.56	10.0	10.1	1.89	2.05	1.01	0.28
C20:0	20.0	34.6	34.2	40.0	35.1	37.7	41.8
C20:1 *c*-9	4.34	67.1	65.4	55.3	44.5	43.7	47.1
C20:1 *c*-11	n.d.	2.02	2.08	0.10	0.54	0.19	0.35
C20:2 n-6	n.d.	62.3	59.3	6.37	4.53	4.75	3.91
C20:3 n-6	n.d.	2.44	3.49	0.60	0.17	0.87	0.33
C20:3 n-3	n.d.	13.4	8.40	4.90	2.89	3.22	2.89
C20:4 n-6	1.11	25.3	24.0	4.28	2.75	2.76	1.89
C20:5 n-3	n.d.	298.8	275.8	47.3	35.6	35.9	30.00
C22:0	11.5	31.9	26.7	27.8	28.4	26.6	26.4
C22:1 n-9	n.d.	8.39	8.30	5.87	4.31	4.63	4.38
C22:5 n-3	n.d.	32.9	29.7	2.15	1.47	1.70	2.88
C22:6 n-3	n.d.	119.3	151.5	35.9	23.7	21.9	19.8
Σ SFA	3866.0	3020.3	3205.5	3357.2	3435.2	3312.8	3538.4
Σ MUFA	1450.0	2961.7	3075.0	2322.1	1794.1	1853.0	2104.5
Σ PUFA	436.6	3186.9	3293.7	666.8	495.8	482.4	502.2
Σ PUFA / Σ SFA	0.11	1.06	1.03	0.20	0.14	0.15	0.14
Σ n-3	26.0	705.8	710.2	120.5	87.9	85.3	77.3
Σ n-6	406.0	2481.2	2583.5	546.3	407.9	397.1	424.9
Σ n-3 / Σ n-6	0.06	0.28	0.27	0.22	0.22	0.21	0.18
TFA	5797.4	9236.9	9655.7	6405.3	5779.0	5702.8	6201.1

Abbreviations: *DM* dry matter, *BSF Hermetia illucens*, *c cis*, *t trans*, *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *TFA* total fatty acids, *n.d.* not detected. Values are reported as mean of duplicate analyses

Somatic indexes, carcass yield and coefficient of fatness
At the end of the trial, twenty-eight fish per treatment (seven fish per tank) were sacrificed by over anaesthesia (MS-222, PHARMAQ Ltd., Fordingbridge, Hampshire, UK; 500 mg/L). The fish were individually weighed and then slaughtered to calculate the carcass yield (CY), the hepatosomatic index (HSI), the viscerosomatic index (VSI), and the coefficient of fatness (CF). The following formulas were used:

- CY (%) = [total weight without gut and gonad (g) / fish weight (g)] × 100
- HSI (%) = [liver weight (g) / fish weight (g)] × 100
- VSI (%) = [gut weight (g) / fish weight (g)] × 100
- CF (%) = [perivisceral fat weight (g) / fish weight (g)] × 100.

Physical characteristics, proximate composition and fatty acid profile of fillets
Nine fish per treatment were filleted, and the right fillets were weighed, packaged in a plastic bag and then refrigerated at + 4 °C. After 24 h at + 4 °C, the right fillets were gently dried with paper to remove excess moisture, and then weighed. Subsequently, the muscle pH ($pH_{24\ h}$) and flesh color were assessed on the inside portion of the cranial, medial and caudal region of each fillet. The pH_{24} measurement was performed using a Crison MicropH 2001 (Crison Instruments, Barcelona, Spain) equipped with a combined electrode and an automatic temperature compensator. The flesh color was analysed using a bench colorimeter Chroma Meter CR-400 (Konica Minolta Sensing Inc., Osaka, Japan). The results were expressed in terms of lightness (L*), redness (a*) and yellowness (b*) in the CIELAB color space model [28].

The water holding capacity was calculated as follows:

- Drip loss (DL; %) = [(raw fillet weight (g) − raw fillet weight after 24h (g)) / raw fillet weight (g)] × 100

The fillets were then individually vacuum-packaged in a plastic bag and stored at − 20 °C. After total freezing, the fillets were thawed at + 4 °C, removed from the bags, dried with paper, and weighed to calculate the thawed loss (TL) as follows:

- Thawing loss (TL; %) = [(raw fillet weight (g) − thawed fillet weight (g)) / raw fillet weight (g)] × 100

The same fillets were then vacuum-packaged in a plastic bag and cooked in a fish kettle for 10 min at 80 °C (core temperature of the fillets: 75 °C). After cooking, the bags were removed from the fish kettle and cooled in fresh water for 15 min to stop the cooking process. Then, the fillets were removed from the bags, dried with paper and weighed again to calculate the cooking loss (CL), as follows:

- Cooking loss (CL; %) = [(raw fillet weight (g) − cooked fillet weight (g)) / raw fillet weight (g)] × 100

Following cooking loss determination, a cooked fish sample (1.5 cm × 1.5 cm) from each fillet was sheared perpendicular to the fibre direction using the Instron 5543 Universal Testing Machine (Instron Corporation, Canton, Massachusetts, USA) equipped with a straight edged shear blade (crosshead speed of 30 mm/min). The maximum peak force recorded during the analysis was reported as Newton (N) shear force.

The nine left fillets per treatment were frozen, finely ground with a knife mill (Grindomix GM200; Retsch GmbH, Haan, Germany) and freeze-dried (Edwards MF 1000, Milan, Italy) to determine their proximate composition (DM, CP, EE, and ash), according to the same procedures implemented for feed analyses [23, 24]. The freeze-dried and ground samples of the fish fillet were also used to assess their FA composition. After dichloromethane-methanol extraction of total lipids from fillets, a basic saponification and a BF_3 esterification were used for the determination of the fatty acid composition, adding tridecanoic acid as internal standard, as reported by Renna et al. [29]. FAME were separated using the same analytical instruments and temperature program previously reported for the FA analysis of feeds. Peaks were identified by injecting pure FAME standards as reported in Renna et al. [30]. The results were expressed as mg/100 g wet weight (ww). All chemical analyses were performed in duplicate.

Morphometric investigation
Eight fish per treatment were submitted to morphometric and histopathological evaluation. Samples of anterior and posterior gut were excised and flushed with 0.9% saline solution to remove all the content. Liver samples were also collected. The collected samples were fixed in 10% buffered formalin solution, routinely embedded in paraffin wax blocks, sectioned at 5 μm thickness, mounted on glass slides and stained with Haematoxylin & Eosin. One slide per each intestinal segment was examined by light microscopy and captured with a Nikon DS-Fi1 digital camera (Nikon Corporation, Minato, Tokyo, Japan) coupled to a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germania) using 2.5× objective lens. NIS-Elements F software was used for image capturing.

Morphometric analysis was performed by Image®-Pro Plus software (6.0 version, Media Cybernetics, Maryland,

USA) on 10 well-oriented and intact villi chosen from each gut segment. The evaluated morphometric index was villus height (Vh, from the villus tip to the crypt bottom). The observed histopathological findings were evaluated in all the organs using a semi-quantitative scoring system as follows: absent (score = 0), mild (score = 1), moderate (score = 2) and severe (score = 3). Gut histopathological findings were separately assessed for mucosa (inflammatory infiltrates) and submucosa (inflammatory infiltrates and Gut-Associated Lymphoid Tissue activation) for each segment. The total score of each gut segment was obtained by adding up the mucosa and submucosa scores. All the slides were blind assessed by two independent observers and the discordant cases were reviewed, using a multi-head microscope, until unanimous consensus was reached.

Digestibility trial

In parallel with the growth trial, an *in vivo* digestibility experiment was performed to determine the apparent digestibility coefficients (ADC) of the diets. Two hundred and forty rainbow trout (mean individual body weight: 100.6 ± 8.53 g) were divided into twelve 250-L cylindro-conical tanks (two replicates per treatment, twenty fish per tank) connected to the same open water system of the growth trial. After 14 days of acclimatization with the experimental diets, the fish were fed by hand to visual satiation two times per day (at 8:00 and 15:00 h), six days per week. The feces were collected daily from each tank for three consecutive weeks, using a continuous automatic device (Choubert' system) as described by Palmegiano et al. [31]. To ensure the correct level of replications per treatment (i.e. $n = 4$), the experiment was conducted over two blocked events using the same batch of fish for both blocks. Before new fecal collection commenced, the fish were allowed to acclimatize to their new diet for a period of 10 days [32]. The feces were pooled within tank, kept frozen (− 20 °C) before being freeze dried and then refrigerated (+ 4 °C) until analysed. The ADCs were measured using the indirect acid-insoluble ash method. For this purpose, the fish were fed the same experimental diets of the growth trial, added with 1% Celite® (Fluka, St. Gallen, Switzerland) as an inert marker in substitution of 1% of wheat gluten meal. The ADC of DM, CP, EE, and GE were calculated as reported by Caimi et al. [19] and expressed as a percentage.

Statistical analyses

Data were analysed using IBM SPSS Statistics v. 25.0 for Windows. One way-ANOVA or Kruskall Wallis tests were used to compare data among the experimental groups. The assumption of normality was checked using the Kolmogorov–Smirnov test. The assumption of homoscedasticity was assessed by Levene's homogeneity of variance test. If such an assumption did not hold, the Brown-Forsythe statistic was applied to test the equality of group means instead of the F one. Pairwise multiple comparisons were performed to test the difference between each pair of means (Tukey's test and Tamhane's T2 in the cases of equal variances assumed or not assumed, respectively [one-way ANOVA], or Dunn's test [Kruskall-Wallis test]). The results were expressed as the mean and pooled standard error of the mean (SEM) or median and interquartile range (IR), depending on data distribution. Significance was set at $P < 0.05$.

Results

Diets

As expected, the DM, ash, CP, EE and GE contents were comparable among the experimental diets (Table 1). Table 2 shows the AA composition of the BSF meal and the experimental diets. Leucine, valine and tyrosine were the most represented essential AA (EAA). Excepted for histidine, methionine and tyrosine, all EAA decreased with the increase of BSF inclusion. However, all diets covered the fish AA requirements. As far as the FA composition is concerned (Table 3), the concentration of total SFA increased at the increase of the insect meal in the diet. In particular, C12:0 showed a 42-fold higher concentration in BSF15 than in BSF0. Noticeable decreases were observed for total PUFA. Omega-3 FA drastically decreased and reached the absolute lowest concentration in the BSF15 diet.

Growth performance

The mortality ranged from 0 (BSF0 and BSF12) to 2.1% (BSF15) and was not affected by the dietary treatment (Table 4). No differences were observed for the considered growth performance traits ($P > 0.05$).

Somatic indexes, carcass yield and coefficient of fatness

The results concerning the somatic indexes, carcass yield and coefficient of fatness are reported in Table 5. The HSI showed an increasing trend with the increase of BSF meal inclusion in the diet. Specifically, HSI values were higher in BSF15 fish than BSF0 and BSF3 fish, while the other treatments showed intermediate values ($P < 0.001$). A similar trend was also observed for VSI, except for BSF0 that showed comparable values as BSF6, BSF9 and BSF12 ($P < 0.01$).

Physical characteristics, proximate composition and fatty acid profile of fillets

The dietary treatment did not affect either color or pH_{24} of the fillets (Table 6). The fillet's physical characteristics were also unaffected by diet (Table 7).

Table 4 Effect of the dietary inclusion of BSF meal on growth performance of rainbow trout ($n = 4$)

	BSF0	BSF3	BSF6	BSF9	BSF12	BSF15	SEM	*P*-value
Mortality, %	0.00	1.04	1.04	1.04	0.00	2.08	0.512	0.708
iIBW, g	100.1	100.1	100.4	100.0	100.4	100.0	0.388	0.999
iFBW, g	278.8	282.3	284.9	278.4	272.9	267.0	2.06	0.179
iWG, g	178.8	182.3	184.6	178.5	172.6	167.3	3.536	0.818
SGR, %/d	0.84	0.84	0.84	0.83	0.82	0.78	0.010	0.675
FCR	1.08	1.09	1.09	1.12	1.13	1.18	0.013	0.398
PER	2.02	1.99	2.00	1.94	1.94	1.83	0.024	0.339

Abbreviations: BSF Hermetia illucens, SEM standard error of the mean, *iIBW* individual initial body weight, *iFBW* individual final body weight, *iWG* individual weight gain, *SGR* specific growth rate, *FCR* feed conversion ratio, *PER* protein efficiency ratio

The DM, CP and EE contents of the fillets did not show differences among the dietary treatments. The ash content was higher in BSF0 compared to BSF9, BSF12 and BSF15, while the fish fed with BSF3 and BSF6 showed intermediate values.

As for FA contents, total SFA and total MUFA showed a clear increasing trend, while a decreasing trend was observed for total PUFA, as the level of BSF larva meal increased in the diet (Table 8). Consequently, the Σ PUFA / Σ SFA ratio of the fillets progressively decreased, ranking in the following order: BSF0 = BSF3 = BSF6 > BSF9 > BSF12 > BSF15. The fish fed with BSF15 showed a higher content of total SFA when compared to the fish fed with BSF0 (+ 29%), while the fish fed with the other treatments showed intermediate values ($P < 0.05$). Regarding individual SFA, C12:0 was about 19-fold higher in BSF15 when compared to BSF0 (1.73 vs. 0.09 g/100 g TFA, respectively; $P < 0.001$). An increasing trend was also observed for C14:0 with the dietary increase of the insect meal, while other individual SFA (i.e., C16:0, C17:0, C18:0 and C20:0) only showed higher values in BSF15 when compared to the other treatments. Various branched chain fatty acids (BCFA) were detected. The rates of all of them, with the exception of C18 *iso*, were significantly affected by the diet. The majority of BCFA showed the absolute highest content when the fish were fed the BSF15 diet. Total MUFA and C18:1 *c*-9 were about 1.3-fold higher in BSF15 when compared to BSF0. As previously described for individual SFA and BCFA, some individual MUFA (i.e., C14:1 *c*-9 – which coeluted with C15:0 in the chromatograms –, total C18:1 *t* and C18:1 *c*-11) showed higher values in BSF15 when compared to the other treatments. The observed decrease of total PUFA was substantial (– 58% in BSF15 when compared to BSF0) and regarded both total n-3 and total n-6 FA ($P < 0.001$). The decrease was more marked for FA belonging to the n-3 (– 81% considering total n-3 FA) than the n-6 (– 43% considering total n-6 FA) series. Consequently, the Σ n-3 / Σ n-6 FA ratio also significantly decreased while increasing BSF meal in the diet, following the order BSF0 > BSF3 = BSF6 = BSF9 > BSF12 > BSF15. All individual n6 FA showed significantly lower values in BSF15 when compared to the other treatments. Similar results were obtained for C18:3 n-3 and C20:3 n-3, while the decreasing trend was gradual for long-chain n-3 PUFA (C20:5 n-3, C22:5 n-3 and C22:6 n-3).

Morphometric investigation

No differences were found for morphometry at the anterior gut, whereas villi were higher in the posterior gut of BSF6 and BSF9 groups when compared to BSF3 (Table 9).

Regarding the histopathological alterations of liver, absent to mild multifocal lymphoplasmacytic inflammatory infiltrates were observed with absent/mild vacuolar degeneration (Table 10). The anterior and posterior gut showed from absent to mild mucosal lymphoplasmacytic infiltration. However, BSF meal inclusion did not affect the severity of the observed histopathological alterations ($P > 0.05$).

Table 5 Effect of the dietary inclusion of BSF meal on somatic indexes, carcass yield and coefficient of fatness of rainbow trout ($n = 28$)

	BSF0	BSF3	BSF6	BSF9	BSF12	BSF15	SEM	*P*-value
HSI	1.20^{b}	1.22^{b}	1.37^{ab}	1.37^{ab}	1.39^{ab}	1.47^{a}	0.019	0.000
VSI	11.2^{ab}	11.0^{b}	11.6^{ab}	12.1^{ab}	12.3^{ab}	12.6^{a}	0.143	0.008
CY	87.9	87.7	87.7	87.0	86.8	89.1	0.341	0.448
CF	3.65	3.64	3.64	3.82	3.84	3.24	0.093	0.622

Abbreviations: BSF Hermetia illucens, SEM standard error of the mean, *HSI* hepatosomatic index, *VSI* viscerosomatic index, *CY* carcass yield, *CF* coefficient of fatness. Different superscripts within a row indicate significant differences ($P < 0.05$)

Table 6 Effect of the dietary inclusion of BSF meal on flesh color and pH_{24} of cranial, medial and caudal regions of rainbow trout fillets (*n* = 9)

	BSF0	BSF3	BSF6	BSF9	BSF12	BSF15	SEM	*P*-value
Cranial region								
L*	42.3	46.1	43.5	45.0	43.2	43.4	0.799	0.788
a*	0.35	0.42	−0.32	2.06	−0.35	0.33	0.255	0.069
b*	6.25	8.06	6.32	8.17	6.17	6.60	0.258	0.051
pH_{24}	6.49	6.31	6.38	6.32	6.37	6.42	0.020	0.101
Medial region								
L*	42.0	45.1	44.3	47.2	43.6	45.0	0.778	0.562
a*	1.57	3.73	1.58	2.70	2.88	1.87	0.316	0.292
b*	7.93	9.66	8.26	9.86	7.60	8.51	0.314	0.204
pH_{24}	6.40	6.26	6.33	6.28	6.31	6.35	0.019	0.119
Caudal region								
L*	44.3	49.6	47.1	47.0	45.8	47.3	0.743	0.490
a*	2.95	2.14	2.93	3.92	4.81	2.31	0.579	0.784
b*	9.08	10.30	9.68	10.54	7.98	8.60	0.337	0.203
pH_{24}	6.37	6.26	6.29	6.25	6.28	6.34	0.017	0.143

*Abbreviations: BSF Hermetia illucens, L** lightness, *a** redness, *b** yellowness, *SEM* standard error of the mean

Digestibility trial

The apparent digestibility of DM, CP, EE and GE was not influenced by diet ($P > 0.05$) (Table 11). The ADC of DM ranged between 86.8% (BSF6) and 87.7% (BSF9). Overall, the ADC of CP and EE were higher than 94% and 98%, respectively. Results concerning ADC of GE ranged between 92.1% (BSF9) and 93.1% (BSF15).

Discussion

Growth performance

Starting from the first day of trial, all the fish promptly accepted all the experimental diets. After 131 days of trial, there were no significant differences among the groups in terms of growth performance and feed utilization. The results obtained in the present study showed that, in current typical commercial diets for trout, it is possible to substitute up to the 50% of FM with a partially defatted BSF larva meal (corresponding to a maximum of 15% dietary inclusion level) without adverse effects on the fish growth performance. Such results are consistent with previous trials in which other fish species were fed diets with inclusion levels up to 20% of full-fat [33], partially defatted [21, 34] and highly defatted BSF meals [19, 35]. Indeed, inclusion levels of BSF meal higher than 20% can induce a stress response in rainbow trout [36]. Published studies show that dietary inclusion levels of about 33% and 26% of full-fat BSF meals [16, 37] or of a partially defatted BSF meal [15] determined a worsening of the WG and feed utilization. Similar results were also observed in meagre (*Argyrosomus regius*) when fed increasing amounts of a partially defatted BSF meal [20]. However, other trials showed that it could be possible to include up to 40% of a partially defatted BSF larva meal [14] or up to 50% of a full-fat BSF prepupae meal [13] in substitution of FM, without negative effects on the growth performance of adult and juvenile rainbow trout, respectively.

Somatic indexes, carcass yield and coefficient of fatness

In literature, HSI values between 1% and 2% (as obtained in the current trial) are indicators of proper liver function [38]. Published data on the effect of insect meals on HSI values in rainbow trout are inconsistent. Similarly to what obtained in our trial, Dumas et al. [15] and

Table 7 Effect of the dietary inclusion of BSF meal on the physical characteristics of rainbow trout fillets (*n* = 9)

	BSF0	BSF3	BSF6	BSF9	BSF12	BSF15	SEM	*P*-value
Drip loss, %	2.89	3.32	2.76	3.22	2.80	3.04	0.114	0.658
Thawed loss, %	9.50	9.47	8.98	10.52	10.18	9.35	0.240	0.470
Cooking loss, %	16.6	17.6	16.6	18.8	18.3	17.7	0.373	0.439
Shear force, N	28.8	26.0	21.1	25.8	26.7	22.71	1.142	0.434

Abbreviations: BSF Hermetia illucens, SEM standard error of the mean

Table 8 Effect of the dietary inclusion of BSF meal on fillet proximate composition (g/100 g ww) and fatty acid profile (g/100 g of TFA) of rainbow trout ($n = 9$)

	BSF0	BSF3	BSF6	BSF9	BSF12	BSF15	SEM	*P*-value
Proximate composition								
DM	25.8	26.8	27.2	25.5	26.1	26.6	0.303	0.643
CP	19.2	20.0	18.3	19.2	19.6	20.5	0.323	0.493
EE	4.44	4.97	5.34	4.74	5.13	4.76	0.223	0.897
Ash	2.06^{a}	1.68^{ab}	1.54^{ab}	1.43^{b}	1.28^{b}	1.22^{b}	0.065	0.010
Fatty acid composition								
C12:0	0.09^{e}	0.28^{de}	0.44^{d}	0.75^{c}	1.16^{b}	1.73^{a}	0.081	0.000
C13 *iso*	0.07^{ab}	0.07^{ab}	0.06^{ab}	0.07^{a}	0.05^{ab}	0.04^{b}	0.003	0.024
C14:0	2.93^{c}	3.00^{bc}	2.91^{c}	3.16^{bc}	3.35^{b}	4.36^{a}	0.078	0.000
C15 *iso*	0.07^{b}	0.07^{b}	0.07^{b}	0.06^{b}	0.07^{b}	0.09^{a}	0.001	0.000
C15 *aiso*	0.02^{b}	0.02^{b}	0.02^{b}	0.02^{b}	0.03^{ab}	0.04^{a}	0.002	0.000
C14:1 *c*-9 + C15:0	0.33^{b}	0.33^{b}	0.32^{b}	0.34^{b}	0.34^{b}	0.43^{a}	0.007	0.000
C16:0	18.6^{b}	19.1^{b}	18.8^{b}	19.6^{b}	20.2^{b}	25.4^{a}	0.361	0.000
C17 *iso*	0.21^{b}	0.22^{b}	0.21^{b}	0.20^{b}	0.22^{b}	0.28^{a}	0.005	0.000
C17 *aiso*	0.43^{cd}	0.42^{d}	0.42^{d}	0.50^{bc}	0.52^{b}	0.64^{a}	0.013	0.000
C16:1 *c*-9	4.88^{c}	5.14^{bc}	5.03^{bc}	5.29^{bc}	5.52^{b}	6.31^{a}	0.085	0.000
C17:0	0.29^{b}	0.28^{b}	0.29^{b}	0.27^{b}	0.28^{b}	0.40^{a}	0.008	0.000
C18 *iso*	0.12	0.11	0.10	0.12	0.12	0.12	0.004	0.421
C17:1 *c*-9	0.19	0.18	0.20	0.23	0.21	0.21	0.006	0.217
C18:0	5.11^{b}	5.13^{b}	5.10^{b}	5.32^{b}	5.44^{b}	6.53^{a}	0.078	0.000
C18:1 *t*-6–10	0.09^{b}	0.10^{b}	0.09^{b}	0.10^{b}	0.11^{ab}	0.12^{a}	0.002	0.000
C18:1 *t*-11–12	0.03	0.03	0.02	0.02	0.02	0.03	0.001	0.104
C18:1 *t*-13–14 + *c*-6–8	0.12^{b}	0.12^{b}	0.11^{b}	0.13^{b}	0.12^{b}	0.16^{a}	0.003	0.000
C18:1 *c*-9	23.3^{d}	24.0^{cd}	24.9^{bcd}	25.4^{bc}	26.6^{b}	31.3^{a}	0.397	0.000
C18:1 *c*-11	2.88^{b}	2.93^{b}	2.92^{b}	2.93^{b}	2.99^{b}	3.50^{a}	0.038	0.000
C18:2 n-6	21.0^{a}	21.6^{a}	21.8^{a}	20.9^{a}	20.7^{a}	12.3^{b}	0.513	0.000
C18:3 n-3	2.51^{a}	2.62^{a}	2.51^{a}	2.41^{a}	2.22^{a}	0.93^{b}	0.089	0.000
C18:3 n-6	0.32^{a}	0.31^{a}	0.34^{a}	0.36^{a}	0.29^{a}	0.12^{b}	0.014	0.000
C20:0	0.16^{b}	0.17^{b}	0.18^{b}	0.18^{b}	0.18^{b}	0.23^{a}	0.004	0.000
C20:1 *c*-9	1.17^{b}	1.15^{b}	1.20^{b}	1.22^{b}	1.26^{ab}	1.44^{a}	0.024	0.003
C20:1 *c*-11	0.17^{a}	0.16^{ab}	0.15^{ab}	0.15^{ab}	0.14^{b}	0.05^{c}	0.006	0.000
C20:2 n-6	0.98^{a}	0.93^{a}	1.01^{a}	0.96^{a}	0.95^{a}	0.66^{b}	0.025	0.000
C20:3 n-3	0.15^{a}	0.15^{a}	0.16^{a}	0.14^{a}	0.14^{a}	0.07^{b}	0.005	0.000
C20:3 n-6	0.63^{a}	0.59^{a}	0.64^{a}	0.70^{a}	0.60^{a}	0.20^{b}	0.025	0.000
C20:4 n-6	0.75^{a}	0.72^{a}	0.73^{a}	0.76^{a}	0.62^{a}	0.22^{b}	0.031	0.000
C20:5 n-3	2.96^{ab}	3.14^{a}	2.68^{ab}	2.53^{b}	1.82^{c}	0.54^{d}	0.128	0.000
C22:0	0.10	0.10	0.12	0.12	0.11	0.11	0.002	0.055
C22:1 n-9	0.14	0.14	0.15	0.16	0.17	0.17	0.006	0.517
C22:5 n-3	0.92^{ab}	1.03^{a}	0.92^{ab}	0.80^{b}	0.55^{c}	0.12^{d}	0.046	0.000
C22:6 n-3	8.19^{a}	5.56^{b}	5.35^{bc}	4.18^{cd}	2.97^{d}	1.11^{e}	0.326	0.000
Σ SFA	27.7^{b}	28.4^{ab}	28.2^{ab}	29.7^{ab}	31.0^{ab}	35.7^{a}	0.821	0.043
Σ BCFA	0.91^{bc}	0.91^{bc}	0.86^{c}	0.97^{b}	1.00^{b}	1.22^{a}	0.018	0.000
Σ MUFA	33.0^{d}	34.0^{cd}	34.8^{bcd}	35.6^{bc}	37.2^{b}	43.3^{a}	0.519	0.000

Table 8 Effect of the dietary inclusion of BSF meal on fillet proximate composition (g/100 g ww) and fatty acid profile (g/100 g of TFA) of rainbow trout ($n = 9$) *(Continued)*

	BSF0	BSF3	BSF6	BSF9	BSF12	BSF15	SEM	*P*-value
Σ PUFA	38.4^{a}	36.7^{ab}	36.1^{ab}	33.8^{bc}	30.8^{c}	16.3^{d}	1.089	0.000
Σ PUFA / Σ SFA	1.39^{a}	1.29^{a}	1.28^{a}	1.14^{b}	1.00^{c}	0.39^{d}	0.047	0.000
Σ n-3	14.7^{a}	12.5^{b}	11.6^{bc}	10.1^{c}	7.71^{d}	2.78^{e}	0.559	0.000
Σ n-6	23.7^{a}	24.2^{a}	24.5^{a}	23.7^{a}	23.1^{a}	13.5^{b}	0.590	0.000
Σ n-3 / Σ n-6	0.62^{a}	0.51^{b}	0.47^{b}	0.43^{b}	0.34^{c}	0.19^{d}	0.020	0.000

Abbreviations: BSF Hermetia illucens, ww wet weight, *TFA* total fatty acid, *SEM* standard error of the mean, *DM* dry matter, *CP* crude protein, *EE* ether extract, *c cis*, *t trans*, *SFA* saturated fatty acids, *BCFA* branched chain fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids. Values are reported as mean of duplicate analyses. Different superscripts within a row indicate significant differences ($P < 0.05$)

Chemello et al. [22] also reported an increase of HSI in rainbow trout fed with partially defatted BSF and *Tenebrio molitor* meals, respectively. On the contrary, Sealey et al. [37] recorded a decrease in HSI in fish fed with diets characterized by increasing levels of BSF meal. Renna et al. [14] observed that inclusion levels up to 40% of a partially defatted BSF larva meal did not influence the HSI in rainbow trout. The dissimilarity of results found in literature could be related to several factors, such as the insect species and the nutritional composition of the insect meal, whereas the chemical composition of BSF meal, especially its fat content and FA profile, could be influenced by the rearing substrate of the larva [9, 39, 40] and the defatting method [15, 41]. In addition, results can also differ due to fish species, size and physiological status.

Physical characteristics, proximate composition and fatty acid profile of fillets

The pH of fish fillets provides a measure of the stress status of the fish and the flesh freshness. Changes in pH could be due by multiple factors, such as high density in tank, stress in pre-killing phase and dietary treatments [42]. An increase of the pH during storage could also reflect the production of bacterial metabolites and therefore a decrease of the shelf life of the product [43]. In the currently trial, the pH of the fillets was not affected by diet, and the recorded values are in line with those previously reported for rainbow trout fed with BSF larva meals [14, 44] and *Tenebrio molitor* larva meals [38]. The results obtained in our study also showed that an inclusion of partially defatted BSF meal up to 15% did not influence the color of the raw fillets. Such results confirm those recently obtained by Secci et al. [44] in rainbow trout fed up to 40% of partially defatted BSF larva meal. The lack of influence of BSF meal on fillet color should be considered as an advantage. In fact, color is an important quality parameter of the fillet, as it directly influences the perception of freshness, being also used by consumers to evaluate product quality [45].

We observed no differences in terms of fillets DL, TL, CL and SF among the experimental groups. Similarly, Secci et al. [44] and Borgogno et al. [46] reported no significant variations for the SF and CL when rainbow trout were fed with BSF meals. In our trial, both SF and CL showed higher values when compared to those obtained by the above-mentioned authors. Such difference may be due to different factors, such as sampling procedure, preservation and storage of samples and analytical methods used.

The lack of effects of BSF meal on fillet DM, CP and EE contents confirms previous findings obtained by Renna et al. [14], Mancini et al. [47] and Reyes et al. [48], when BSF larva meals were included in rainbow trout diets up to inclusion levels of 25%. In our trial, the ash content of the fillets showed a decreasing trend following increasing levels of BSF larva meal inclusion in the diet. When compared to FM, BSF meal showed noticeably lower phosphorous levels [49]. Such difference may have determined the observed significant decreases in fillet ash content at increasing BSF levels in the diet. However, in young grass carp (*Ctenopharyngodon idella* Valenciennes), Wen et al. [50] observed decreased levels of ash in fillets while increasing the dietary level of available phosphorous. As no information can be found in literature on rainbow trout, further studies are needed to clearly understand the obtained results.

Fish contain high amounts of long-chain n-3 PUFA, well known to exert beneficial effects on human health [51]. Usually, the FA composition of fish fillets reflects

Table 9 Effect of the dietary inclusion of BSF meal on morphometric traits of anterior and posterior gut of rainbow trout ($n = 8$)

	BSF0	BSF3	BSF6	BSF9	BSF12	BSF15	SEM	*P*-value
Vh anterior gut	0.54	0.51	0.54	0.50	0.54	0.49	0.015	0.906
Vh posterior gut	0.66^{abc}	0.53^{c}	0.66^{a}	0.63^{ab}	0.63^{abc}	0.53^{bc}	0.013	0.007

Abbreviations: BSF Hermetia illucens, SEM standard error mean, *Vh* villus height. Different superscripts within a row indicate significant differences ($P < 0.05$)

Table 10 Effect of the dietary inclusion of BSF meal on histopathological alterations of liver and gut of rainbow trout ($n = 8$)

	BSF0	BSF3	BSF6	BSF9	BSF12	BSF15	*P*-value
Liver							
Inflammation, median (IR)	0.5 (0.0–1.0)	0.0 (0.0–0.4)	0.0 (0.0–0.0)	0.0 (0.0–1.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.057
Degeneration, median (IR)	0.0 (0.0–0.4)	0.0 (0.0–0.5)	0.25 (0.0–1.0)	1.25 (0.5–1.9)	0.25 (0.0–1.0)	0.0 (0.0–1.0)	0.071
Gut							
Anterior, median (IR)	0.0 (0.0–0.0)	0.0 (0.0–0.5)	0.0 (0.0–0.9)	0.0 (0.0–0.0)	0.0 (0.0–0.8)	0.0 (0.0–0.0)	0.180
Posterior, median (IR)	0.0 (0.0–0.8)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.345

Abbreviations: BSF Hermetia illucens, IR interquartile range

that of the administered diet [14]. The FA content and composition of insect larvae (and derived meals) depend on the considered insect species, rearing substrate, developmental stage and processing (e.g., defatting methods) [52]. Differently from FM, insects are generally rich in SFA and poor in PUFA [53]. In particular, BSF larval fat consists mainly of C12:0 and other SFA [9, 52], as confirmed by the analyses of the tested BSF meal. Even if the BSF meal used in this trial was partially defatted (EE: 7.0 g/100 g as fed), this led to a noticeable increase of C12:0 in the BSF-containing diets when compared to the FM-control diet (BSF0), as already observed by other authors [14, 20]. In recent trials where high inclusion levels of insect meals were evaluated in aquafeed for various fish species, significant alterations of the FA composition of fish whole body and fish fillets were observed [21, 47, 54]. The most frequently reported modifications were increasing levels of SFA associated with reductions of PUFA contents, particularly when considering long-chain PUFA of the n-3 series (i.e., C20:5n-3 and C22:6n-3), which also led to undesirable decreases of the PUFA/SFA and n-3/n-6 PUFA ratios of the product [19, 20, 44]. Our results confirm such findings and, in addition, clearly show that the FA composition of trout fillets could be negatively affected even in case of low inclusion levels of BSF meal as a replacement of FM in typical commercial diets.

Morphometric investigation

Regardless the dietary treatment, Vh showed a proximo-distal increasing gradient from anterior to posterior gut. In literature, the majority of the studies conducted in mammals and poultry reported a proximo-distal decreasing gradient from anterior to posterior gut [55]. This is due to the different intensity of nutrient digestion and absorption processes along the gut. Only few publications are available on the morphometry of rainbow trout and they mainly concentrated on the anterior gut [56] as it is the most important site for nutrient absorption, receiving physical, chemical and hormonal stimuli caused by the presence of the diet in the lumen [57, 58]. The lack of differences for Vh in the anterior gut among the dietary treatments is a positive finding and it is in accordance with Renna et al. [14] who did not record any morphological changes in the intestine of trout fed up to 40% of BSF meal. Moreover, the unaffected morphometry of the anterior gut could also explain the unchanged growth performances and diet digestibility recorded for the trout of the present study, suggesting good nutrient absorption and utilization with BSF meal dietary inclusion levels up to 15%. On the contrary, BSF meal influenced Vh in the posterior gut, being lower in BSF3 diet compared to the other treatments. This is one of the first studies describing posterior gut in trout and further study are needed to better investigate this upward trend. Regarding the histopathological alterations of liver and gut, they varied from absent to mild in all the organs. The absence of adverse effects related to dietary BSF meal inclusion observed in liver and gut of the present study is in agreement with published literature [36, 59, 60].

Table 11 Apparent digestibility coefficients (ADC) of the experimental diets ($n = 4$)

	BSF0	BSF3	BSF6	BSF9	BSF12	BSF15	SEM	*P*-value
ADC_{DM}, %	87.3	87.1	86.8	87.7	86.9	87.5	0.332	0.988
ADC_{CP}, %	94.7	94.2	94.4	94.6	94.2	94.6	0.163	0.967
ADC_{EE}, %	98.8	98.7	98.7	98.8	98.6	98.6	0.054	0.903
ADC_{GE}, %	92.9	92.5	92.7	92.1	92.6	93.4	0.240	0.947

Abbreviations: BSF Hermetia illucens, SEM standard error of the mean, *DM* dry matter, *CP* crude protein, *EE* ether extract, *GE* gross energy. Different superscript letters within a row indicate significant differences ($P < 0.05$)

Digestibility trial

The ADC values of nutrients and energy were high and comparable among the experimental groups. Such results suggest that BSF larva meal is well digested by rainbow trout and its inclusion up to 15% in the diet does not adversely affect the fish growth performance. In fact, studies conducted on rainbow trout with BSF larva meal [14] and *Tenebrio molitor* larva meal [22, 61], showed that dietary inclusion levels up to 25% did not affect the apparent digestibility of nutrients and energy.

Conclusions

The findings of this study suggest that partially defatted black soldier fly larva meal can be considered a suitable ingredient in low fishmeal-based diets for rainbow trout. In this study, we did not observe any adverse effect on growth performance, somatic indexes, fillet physical quality parameters and chemical composition, and diets digestibility. However, the FA composition of fillets worsened while increasing the level of BSF meal in the diets. In particular, a decrease of PUFA, and an increase of total SFA and total MUFA was observed. The commercial use of insect meal for aquaculture feed is a good approach to reduce the environmental impact and support a sustainable increase in aquaculture production.

Abbreviations

ADC: Apparent digestibility coefficient; ADF: Acid detergent fiber; BCFA: Branched chain fatty acids; BSF: *Hermetia illucens*; *c*: *cis*; CF: Coefficient of fatness; CL: Cooking loss; CP: Crude protein; CY: Carcass yield; DL: Drip loss; DM: Dry matter; EE: Ether extract; FA: Fatty acid; FAME: Fatty acid methyl ester; FBW: Final body weight; FCR: Feed conversion ratio; FM: Fish meal; GE: Gross energy; HSI: Hepatosomatic index; IBW: Initial body weight; IR: Interquartile range; MUFA: Monounsaturated fatty acids; nd: Not detected; PAPs: Processed animal proteins; PER: Protein efficiency ratio; PUFA: Polyunsaturated fatty acids; SEM: Standard error of the mean; SF: Shear force; SFA: Saturated fatty acids; SGR: Specific growth rate; *t*: *trans*; TFA: Total fatty acids; TL: Thawed loss; Vh: Villus height; VSI: Viscerosomatic index; WG: Weight gain; ww: Wet weight

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Authors' contributions

CC, IB, AS and LG conceived and designed the experiment. CC, IB, GC, SBO, FG, AS, AT, MR and LG prepared the diets, performed the growth and digestibility trial and collected the experimental data. AB and CC performed the fillet physical quality parameters. CC, CL, VM and AT carried out the chemical analyses. MTC and EC performed the histomorphometric investigations and analysed the data. CC, AB, IB and MR performed the statistical analysis. CC, FG, MR and LG analysed and interpreted the data. CC, MR and LG wrote the first draft of the manuscript. All the authors critically reviewed the manuscript for its intellectual content and gave their approval for the final version to be published.

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Availability of data and materials

The datasets analysed in the current study are available from the corresponding author on request.

Declarations

Ethics approval and consent to participate

The experimental protocol was approved by the Ethical Committee of the University of Turin (Italy) (protocol n° 143811).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Department of Agricultural, Forest and Food Sciences, University of Turin, Largo P. Braccini 2, 10095 Grugliasco, TO, Italy. [2]Department of Veterinary Sciences, University of Turin, Largo P. Braccini 2, 10095 Grugliasco, TO, Italy. [3]Institute of Science of Food Production, National Research Council, Largo P. Braccini 2, 10095 Grugliasco, TO, Italy. [4]Department of Comparative Biomedicine and Food Science, University of Padua, Viale dell'Università 16, 35020 Legnaro, PD, Italy.

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References

1. Smárason BÖ, Alriksson B, Jóhannsson R. Safe and sustainable protein sources from the forest industry – the case of fish feed. Trends Food Sci Technol. 2019;84:12–4. https://doi.org/10.1016/j.tifs.2018.03.005.
2. FAO (Food and Agriculture Organization of the United Nation). The state of World Fisheries and Aquaculture 2020. Sustainability in action. Rome: Food and Agriculture Organization of the United Nations (FAO); 2020. https://doi.org/10.4060/ca9229en.
3. Engle RC, D'Abramo L, Ponniah AG, Slater M. Global Aquaculture 2050. J World Aquacult Soc. 2018;48(1):3–6. https://doi.org/10.1111/jwas.12400.
4. Olsen RL, Hasan MR. A limited supply of fishmeal: impact on future increases in global aquaculture production. Trends Food Sci Technol. 2012; 27(2):120–8. https://doi.org/10.1016/j.tifs.2012.06.003.
5. Vongvichith B, Morioka S, Sugita T, Phousavanh N, Phetsanghanh N, Chanthasone P, et al. Evaluation of the efficacy of aquaculture feeds for the climbing perch *Anabas testudineus*: replacement of fishmeal by black soldier fly *Hermetia illucens* prepupae. Fish Sci. 2020;86(1):145–51. https://doi.org/10.1007/s12562-019-01381-5.
6. Smetana S, Schmitt E, Mathys A. Sustainable use of *Hermetia illucens* insect biomass for feed and food: Attributional and consequential life cycle assessment. Resour Conserv Recycl. 2019;144:285–96. https://doi.org/10.1016/j.resconrec.2019.01.042.
7. Gasco L, Biancarosa I, Liland NS. From waste to feed: a review of recent knowledge on insects as producers of protein and fat for animal feeds. Curr Opin Green Sustain Chem. 2020;23:67–79. https://doi.org/10.1016/j.cogsc.2020.03.003.
8. Meneguz M, Schiavone A, Gai F, Dama A, Lussiana C, Renna M, et al. Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (*Hermetia illucens*) larvae. J Sci Food Agric. 2018;98:5776–84. https://doi.org/10.1002/jsfa.9127.
9. Barragan-Fonseca KB, Dicke M, van Loon JJ. Nutritional value of the black soldier fly (*Hermetia illucens* L.) and its suitability as animal feed – a review. J Insects Food Feed. 2017;3(2):105–20. https://doi.org/10.3920/JIFF2016.0055.
10. Gasco L, Acuti G, Bani P, Dalle Zotte A, Danieli PP, De Angelis A, et al. Insect and fish by-products as sustainable alternatives to conventional animal proteins in animal nutrition. Ital J Anim Sci. 2020;19:360–72. https://doi.org/10.1080/1828051X.2020.1743209.
11. Kogut MH, Arsenault RJ. Editorial: gut health: the new paradigm in food animal production. Front Vet Sci. 2016;3:10–3. https://doi.org/10.3389/fvets.2016.00071.
12. Cardinaletti G, Randazzo B, Messina M, Zarantoniello M, Giorgini E, Zimbelli A, et al. Effects of graded dietary inclusion level of full-fat *Hermetia illucens*

prepupae meal in practical diets for rainbow trout (*Oncorhynchus mykiss*). Animals. 2019;9(5):251. https://doi.org/10.3390/ani9050251.

13. Renna M, Schiavone A, Gai F, Dabbou S, Lussiana C, Malfatto V, et al. Evaluation of the suitability of a partially defatted black soldier fly (*Hermetia illucens* L.) larvae meal as ingredient for rainbow trout (*Oncorhynchus mykiss* Walbaum) diets. J Anim Sci Biotechnol. 2017;8:57. https://doi.org/10.1186/s40104-017-0191-3.
14. Dumas A, Raggi T, Barkhouse J, Lewis E, Weltzien E. The oil fraction and partially defatted meal of black soldier fly larvae (*Hermetia illucens*) affect differently growth performance, feed efficiency, nutrient deposition, blood glucose and lipid digestibility of rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 2018;492:24–34. https://doi.org/10.1016/j.aquaculture.2018.03.038.
15. St-Hilaire S, Sheppard C, Tomberlin J, Irving S, Newton L, McGuire M, et al. Fly prepupae as a feedstuff for rainbow trout, *Oncorhynchus mykiss*. J World Aquacult Soc. 2007;38:59–67. https://doi.org/10.1111/j.1749-7345.2006.00073.x.
16. Zarantoniello M, Bruni L, Randazzo B, Vargas A, Gioacchini G, Truzzi C, et al. Partial dietary inclusion of Hermetia illucens (black soldier Fly) full-fat prepupae in zebrafish feed: biometric, histological, biochemical, and molecular implications. Zebrafish. 2018;15(5):519–32. https://doi.org/10.1089/zeb.2018.1596.
17. Józefiak A, Nogales-Mérida S, Mikołajczak Z, Rawski M, Kierończyk B, Mazurkiewicz J. The utilization of full-fat insect meal in rainbow trout (*Oncorhynchus mykiss*) nutrition: the effects on growth performance, intestinal microbiota and gastrointestinal tract histomorphology. Ann Anim Sci. 2019;19(3):747–65. https://doi.org/10.2478/aoas-2019-0020.
18. Caimi C, Renna M, Lussiana C, Bonaldo A, Gariglio M, Meneguz M, et al. First insights on black soldier Fly (*Hermetia illucens* L.) larvae meal dietary administration in Siberian sturgeon (*Acipenser baerii* Brandt) juveniles. Aquaculture. 2020;515:734539. https://doi.org/10.1016/j.aquaculture.2019.734539.
19. Guerreiro I, Castro C, Antunes B, Coutinho F, Rangel F, Couto A, et al. Catching black soldier fly for meagre: growth, whole-body fatty acid profile and metabolic responses. Aquaculture. 2020;516:734613. https://doi.org/10.1016/j.aquaculture.2019.734613.
20. Stejskal V, Tran HQ, Prokesova M, Gebauer T, Giang PT, Gai F, et al. Partially defatted *Hermetia illucens* larva meal in diet of Eurasian perch (*Perca fluviatilis*) juveniles. Animals. 2020;10:1876. https://doi.org/10.3390/ani10101876.
21. Chemello G, Renna M, Caimi C, Guerreiro I, Oliva-Teles A, Enes P, et al. Partially defatted *Tenebrio molitor* larva meal in diets for grow-out rainbow trout, *Oncorhynchus mykiss* (Walbaum): effects on growth performance, diet digestibility and metabolic responses. Animals. 2020;10(2):229. https://doi.org/10.3390/ani10020229.
22. AOAC International. Official methods of analysis of AOAC international. 16th ed. Gaithersburg: Association of Official Analytical Chemists; 2000.
23. AOAC International. Official methods of analysis of AOAC international. 17th ed. Gaithersburg: Association of Official Analytical Chemists; 2003.
24. Finke MD. Estimate of chitin in raw whole insects. Zoo Biol. 2007;26(2):105–15. https://doi.org/10.1002/zoo.20123.
25. De Marco M, Martínez S, Hernandez F, Madrid J, Gai F, Rotolo L, et al. Nutritional value of two insect larval meals (*Tenebrio molitor* and *Hermetia illucens*) for broiler chickens: apparent nutrient digestibility, apparent ileal amino acid digestibility and apparent metabolisable energy. Anim Feed Sci Technol. 2015;209:211–8. https://doi.org/10.1016/j.anifeedsci.2015.08.006.
26. Dabbou S, Gasco L, Lussiana C, Brugiapaglia A, Biasato I, Renna M, et al. Yellow mealworm (*Tenebrio molitor* L.) larvae inclusion in diets for free-range chickens: effects on meat quality and fatty acid profile. Renew Agr Food Syst. 2020;35(5):571–8. https://doi.org/10.1017/S1742170519000206.
27. Dabbou S, Gai F, Renna M, Rotolo L, Dabbou S, Lussiana C, et al. Inclusion of bilberry pomace in rabbit diets: effects on carcass characteristics and meat quality. Meat Sci. 2017;124:77–83. https://doi.org/10.1016/j.meatsci.2016.10.013.
28. CIE (CommissionInternationale de l'Éclairage). Recommendations on uniform color spaces-color difference equations, psychometric color terms (supplement no. 2 to CIE publication no. 15). Paris: Commission Internationale de l'Éclairage; 1976.
29. Renna M, Brugiapaglia A, Zanardi E, Destefanis G, Prandini A, Moschini M, et al. Fatty acid profile and meat quality of beef from double-muscled Piemontese bulls fed ground flaxseed. Ital J Anim Sci. 2019;18(1):355–65. https://doi.org/10.1080/1828051X.2018.1530958.
30. Renna M, Lussiana C, Cornale P, Fortina R, Mimosi A. Changes in goat milk fatty acids during abrupt transition from indoor to pasture diet. Small Rum Res. 2012;108:12–21. https://doi.org/10.1016/j.smallrumres.2012.06.007.
31. Palmegiano GB, Daprà F, Forneris G, Gai F, Gasco L, Guo K, et al. Rice protein concentrate meal as a potential ingredient in practical diets for rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 2006;258(1–4):357–67. https://doi.org/10.1016/j.aquaculture.2006.04.011.
32. Glencross B, Blyth D, Cheers S, Bourne N, Wade N, Irvin S. A compendium of raw material digestibilities for barramundi, *Lates calcarifer*. Aquac Nutr. 2017;23:1055–64. https://doi.org/10.1111/anu.12473.
33. Abdel-Tawwab M, Khalil RH, Metwally AA, Shakweer MS, Khallaf MA, Abdel-Latif HM. Effects of black soldier fly (*Hermetia illucens* L.) larvae meal on growth performance, organs-somatic indices, body composition, and hemato-biochemical variables of European sea bass, *Dicentrarchus labrax*. Aquaculture. 2020;522:735136. https://doi.org/10.1016/j.aquaculture.2020.735136.
34. Fawole FJ, Adeoye AA, Tiamiyu LO, Ajala KI, Obadara SO, Ganiyu IO. Substituting fishmeal with *Hermetia illucens* in the diets of African catfish (*Clarias gariepinus*): effects on growth, nutrient utilization, haemato-physiological response, and oxidative stress biomarker. Aquaculture. 2020;518:734849. https://doi.org/10.1016/j.aquaculture.2019.734849.
35. Wang G, Peng K, Hu J, Yi C, Chen X, Wu H, et al. Evaluation of defatted black soldier fly (*Hermetia illucens* L.) larvae meal as an alternative protein ingredient for juvenile Japanese seabass (*Lateolabrax japonicus*) diets. Aquaculture. 2019;507:144–54. https://doi.org/10.1016/j.aquaculture.2019.04.023.
36. Elia AC, Capucchio MT, Caldaroni B, Magara G, Dörr AJM, Biasato I, et al. Influence of *Hermetia illucens* meal dietary inclusion on the histological traits, gut mucin composition and the oxidative stress biomarkers in rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 2018;496:50–7. https://doi.org/10.1016/j.aquaculture.2018.07.009.
37. Sealey W, Gaylord T, Barrows F, Tomberlin J, McGuire M, Ross C, et al. Sensory analysis of rainbow trout, *Oncorhynchus mykiss*, fed enriched black soldier fly prepupae, *Hermetia illucens*. J World Aquacult Soc. 2011;42:34–45. https://doi.org/10.1111/j.1749-7345.2010.00441.x.
38. Iaconisi V, Bonelli A, Pupino R, Gai F, Parisi G. Mealworm as dietary protein source for rainbow trout: body and fillet quality traits. Aquaculture. 2018;484:197–204. https://doi.org/10.1016/j.aquaculture.2017.11.034.
39. Villazana J, Alyokhin A. Development of black soldier fly larvae (Diptera: Stratiomyidae) on seafood wastes. J Insects Food Feed. 2019;5(4):313–9. https://doi.org/10.3920/JIFF2019.0008.
40. Truzzi C, Giorgini E, Annibaldi A, Antonucci M, Illuminati S, Scarponi G, et al. Fatty acids profile of black soldier fly (*Hermetia illucens*): influence of feeding substrate based on coffee-waste silverskin enriched with microalgae. Anim Feed Sci Technol. 2020;259:114309. https://doi.org/10.1016/j.anifeedsci.2019.114309.
41. Ravi HK, Vian MA, Tao Y, Degrou A, Costil J, Trespeuch C, et al. Alternative solvents for lipid extraction and their effect on protein quality in black soldier fly (*Hermetia illucens*) larvae. J Clean Prod. 2019;238:117861. https://doi.org/10.1016/j.jclepro.2019.117861.
42. Brinker A, Reiter R. Fish meal replacement by plant protein substitution and guar gum addition in trout feed, part I: effects on feed utilization and fish quality. Aquaculture. 2011;310(3–4):350–60. https://doi.org/10.1016/j.aquaculture.2010.09.041.
43. Abbas KA, Mohamed A, Jamilah B, Ebrahimian M. A review on correlations between fish freshness and pH during cold storage. Am J Biochem Biotechnol. 2008;4(4):416–21. https://doi.org/10.3844/ajbbsp.2008.416.421.
44. Secci G, Mancini S, Iaconisi V, Gasco L, Basto A, Parisi G. Can the inclusion of black soldier fly (*Hermetia illucens*) in diet affect the flesh quality/nutritional traits of rainbow trout (*Oncorhynchus mykiss*) after freezing and cooking? Int J Food Sci Nutr. 2019;70(2):161–71. https://doi.org/10.1080/09637486.2018.1489529.
45. Vanhonacker F, Pieniak Z, Verbeke W. European consumer image of farmed fish, wild fish, seabass and seabream. Aquac Int. 2013;21:1017–33. https://doi.org/10.1007/s10499-012-9609-2.
46. Borgogno M, Dinnella C, Iaconisi V, Fusi R, Scarpaleggia C, Schiavone A, et al. Inclusion of *Hermetia illucens* larvae meal on rainbow trout (*Oncorhynchus mykiss*) feed: effect on sensory profile according to static and dynamic evaluations. J Sci Food Agric. 2017;97(10):3402–11. https://doi.org/10.1002/jsfa.8191.
47. Mancini S, Medina I, Iaconisi V, Gai F, Basto A, Parisi G. Impact of black soldier fly larvae meal on the chemical and nutritional characteristics of

rainbow trout fillets. Animal. 2018;12(8):1672–81. https://doi.org/10.1017/S1751731117003421.

48. Reyes M, Rodríguez M, Montes J, Barroso FG, Fabrikov D, Morote E, et al. Nutritional and growth effect of insect meal inclusion on seabass (*Dicentrarchus labrax*) feeds. Fishes. 2020;5(2):16. https://doi.org/10.3390/fishes5020016.
49. Gasco L, Gai F, Maricchiolo G, Genovese L, Ragonese S, Bottari T, et al. Fishmeal alternative protein sources for aquaculture feeds. In Feeds for the aquaculture sector (pp. 1–28). Cham: Springer; 2018. https://doi.org/10.1007/978-3-319-77941-6_1.
50. Wen J, Jiang W, Feng L, Kuang S, Jiang J, Tang L, et al. The influence of graded levels of available phosphorus on growth performance, muscle antioxidant and flesh quality of young grass carp (*Ctenopharyngodon idella*). Animal Nutrition. 2015;1(2):77–84. https://doi.org/10.1016/j.aninu.2015.05.004.
51. Calder PC. Very long-chain n-3 fatty acids and human health: fact, fiction and the future. Proc Nutr Soc. 2018;77(1):52–72. https://doi.org/10.1017/S0029665117003950.
52. Ewald N, Vidakovic A, Langeland M, Kiessling A, Sampels S, Lalander C. Fatty acid composition of black soldier fly larvae (*Hermetia illucens*) – possibilities and limitations for modification through diet. Waste Manag. 2020;102:40–7. https://doi.org/10.1016/j.wasman.2019.10.014.
53. Stanley-Samuelson DW, Jurenka RA, Cripps C, Blomquist GJ, de Renobales M. Fatty acids in insects: composition, metabolism, and biological significance. Arch Insect Biochem Physiol. 1988;9(1):1–33. https://doi.org/10.1002/arch.940090102.
54. Belghit I, Liland NS, Gjesdal P, Biancarosa I, Menchetti E, Li Y, et al. Black soldier fly larvae meal can replace fish meal in diets of sea-water phase Atlantic salmon (*Salmo salar*). Aquaculture. 2019;503:609–19. https://doi.org/10.1016/j.aquaculture.2018.12.032.
55. Biasato I, De Marco M, Rotolo L, Renna M, Lussiana C, Dabbou S, et al. Effects of dietary Tenebrio molitor meal inclusion in free-range chickens. J Anim Physiol Anim Nutr. 2016;100:1104–12. https://doi.org/10.1111/jpn.12487.
56. Ramos MA, Gonçalves JF, Batista S, Costas B, Pires MA, Rema P, et al. Growth, immune responses and intestinal morphology of rainbow trout (*Oncorhynchus mykiss*) supplemented with commercial probiotics. Fish Shellfish Immunol. 2015;45(1):19–26. https://doi.org/10.1016/j.fsi.2015.04.001.
57. He M, Wang KW, Liang X, Fang J, Geng Y, Chem Z, et al. Effects of dietary vitamin E on growth performance as well as intestinal structure and function of channel catfish (*Ictalurus punctatus*, Rafinesque 1818). Exp Ther Med. 2017;14(6):5703–10. https://doi.org/10.3892/etm.2017.5295.
58. Quin C, Yang L, Zheng W, Yan X, Lu R, Xie D, et al. Effects of dietary glucose and sodium chloride on intestinal glucose adsorption of common carp (*Cyprinus carpio* L.). Biochem Biophys Res Commun. 2018;495(2):1948–55. https://doi.org/10.1016/j.bbrc.2017.12.065.
59. Lock ER, Arsiwalla T, Waagbo R. Insect larvae meal as an alternative source of nutrients in the diet of Atlantic salmon (*Salmo salar*) postsmolt. Aquac Nutr. 2016;22(6):1202–13. https://doi.org/10.1111/anu.12343.
60. Magalhães R, Sánchez-López A, Leal RS, Martínez-Llorens S, Oliva-Teles A, Peres H. Black soldier fly (*Hermetia illucens*) pre-pupae meal as a fish meal replacement in diets for European seabass (*Dicentrarchus labrax*). Aquaculture. 2017;476:79–85. https://doi.org/10.1016/j.aquaculture.2017.04.021.
61. Rema P, Saravanan S, Armenjon B, Motte C, Dias J. Graded incorporation of defatted yellow mealworm (*Tenebrio molitor*) in rainbow trout (*Oncorhynchus mykiss*) diet improves growth performance and nutrient retention. Animals. 2019;9(4):187. https://doi.org/10.3390/ani9040187.

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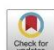

Hermetia illucens meal inclusion in low-fishmeal diets for rainbow trout (*Oncorhynchus mykiss*): Effects on the growth performance, nutrient digestibility coefficients, selected gut health traits, and health status indices

I. Biasato [a,1], G. Chemello [b,1], S. Bellezza Oddon [a], I. Ferrocino [a], M.R. Corvaglia [a], C. Caimi [a], A. Resconi [a], A. Paul [c], M. van Spankeren [c], M.T. Capucchio [d,e], E. Colombino [d], L. Cocolin [a], F. Gai [e,*], A. Schiavone [d,e], L. Gasco [a]

[a] Department of Agricultural, Forest and Food Sciences, University of Turin, Grugliasco, TO, Italy
[b] Department of Life and Environmental Sciences, Marche Polytechnic University, Ancona, Italy
[c] Protix BV, Dongen, the Netherlands
[d] Department of Veterinary Sciences, University of Turin, Grugliasco, TO, Italy
[e] Institute of Sciences of Food Production, National Research Council, Grugliasco, TO, Italy

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ABSTRACT

The effects of including *Hermetia illucens* (HI) meal in rainbow trout diets have been widely characterized, but data related to its utilization in low-fishmeal (FM) diets are quite scarce. The current research investigated the impact of HI meal inclusion in low-FM diets for rainbow trout by assessing fish growth performance, nutrient digestibility, histomorphological traits of intestine and main organs, and gut microbiota. In the 133-days growth trial, 600 rainbow trout were randomly distributed to 4 dietary treatments (3 replicate tanks/treatment, 50 fish/tank): a low-FM diet as control (HI0), and three experimental diets including 80, 160 and 320 g/kg of HI meal as fed as replacement of 25%, 50% and 100% of FM (HI25, HI50 and HI100, respectively). At the end of the trial, growth parameters, condition factor and somatic indices were assessed, and gut, stomach, liver and spleen samples (12 fish/diet) were collected for histomorphological analyses. Feed and posterior intestine content were also sampled to characterize their microbiota. In the digestibility trial, 216 fish (3 tanks/treatment, 18 fish/tank) allowed evaluating the apparent digestibility coefficients (ADC) of the dietary nutrients. Unaffected growth performance, condition factor, somatic indices, nutrient digestibility, and histomorphological features were observed in the HI-fed rainbow trout ($P > 0.05$). Increasing percentages of HI meal in the feeds

Abbreviations: AA, amino acid; ADF, acid detergent fiber; ADC, apparent digestibility coefficient; CF, coefficient of fatness; CY, carcass yield; CP, crude protein; DM, dry matter; EE, ether extract; FA, fatty acid; FAME, fatty acid methyl esters; FCR, feed conversion ratio; FDR, false discovery rate; FM, fishmeal; HE, Haematoxylin & Eosin; HI0, control diet; HI25, *Hermetia illucens* meal as replacement of 25% of fishmeal; HI50, *Hermetia illucens* meal as replacement of 50% of fishmeal; HI100, *Hermetia illucens* meal as replacement of 100% of fishmeal; HSI, hepatosomatic index; iFBW, individual final body weight; iIBW, individual initial body weight; iWG, individual weight gain; NDF, neutral detergent fiber; OTUs, Operational Taxonomic Units; PER, protein efficiency ratio; SCFAs, short-chain fatty acids; SGR, specific growth rate; TFA, total fatty acids; Vh, villus height; VSI, viscerosomatic index.

* Correspondence to: Institute of Sciences of Food Production, National Research Council, Grugliasco, TO 10095, Italy.
E-mail address: francesco.gai@ispa.cnr.it (F. Gai).

[1] B.I. and G.C. contributed equally to this paper.

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determined a progressive increase in the relative abundance of Firmicutes and Actinobacteria phyla, and *Staphylococcus, Enterococcus, Oceanobacillus* and *Actinomyces* genera, whereas Proteobacteria – as well as *Lactobacillus* and *Listeria* – displayed a gradual reduction. The Chao1 index of the fish gut microbiota increased when including HI meal, while the Shannon index displayed the opposite trend ($P < 0.05$). The HI25 and HI50 fish showed enrichment of Actinobacteria, but a reduction in Bacteroidetes (False Discovery Rate [FDR] < 0.05). Furthermore, *Bacillus, Actinomyces, Staphylococcus, Enterococcus,* and *Oceanobacillus* displayed higher relative abundance in the HI-fed fish than the other groups (FDR < 0.05). On the contrary, HI meal utilization was accompanied by a decrease in *Campylobacter* and *Listeria*, as well as *Lactobacillus, Clostridium, Pediococcus, Leuconostoc*, unclassified members (U.m.) of Peptostreptococceae, *Vagococcus, Lactococcus,* and *Weissella* (FDR < 0.05). In conclusion, HI meal can be used in low-FM diets for rainbow trout up to high inclusion levels (32%) without negatively affecting the fish nutrient digestibility, growth performance, somatic indices and histomorphological features. A positive shift of the gut microbiota towards the selection of short-chain fatty acids (SCFAs)-producing bacteria and reduction of foodborne disease-causing pathogens was also observed.

1. Introduction

The commercial rearing of insects for feed production represents a market that has grown rapidly in the recent years, especially in relation to the aquaculture segment (as a reasonable consequence of the early EU authorisation of the insect processed animal proteins [PAPs] in aquaculture feed in July 2017 [IPIFF, 2021]). The rapid development of the insect sector is related to the strong ability of insects to transform the food waste in forms of protein-rich animal feed, thus allowing them to fully embrace the concept of "circular economy". In particular, among the farmed insect species, the black soldier fly (*Hermetia illucens*, HI) represents the most popular choice for mass production, because of its short life cycle, better feed conversion ratio (FCR), and the efficiency in bioconversion (50–60%) and recovery of nutrients from a wide spectrum of organic materials (Ojha et al., 2020). However, the growth of the insect market is strictly connected with two important challenges, such as the meet of consumer's expectations (in terms of consumption of safe, nutritious, and high-quality products) and the update of the regulatory framework (as no animal-based foodstuff can be used to feed insects, with the exception of the ones listed in the Reg. (EU) 2021/1372). In order to overcome these barriers (and, accelerate the scale up process), the insect producers need to currently test their products in the experimental setup.

In order to assess if a novel feed ingredient (such as insect-based products) can be suitable for fish feeding, a two-way approach is commonly adopted. First, the nutritional profile of the feed source needs to be fully characterized, as well as the feed acceptance, the growth performance and the nutrient digestibility by the fish (Rawski et al., 2020). Secondly, the implications for animal health must be investigated, with the attention being mainly directed towards the role of the gut. Indeed, the health status of the intestine (in terms of morphological development, mucin production, and microbiota/microbiome) is fundamental to guarantee a proper health and growth of the fish (Józefiak et al., 2019; Caimi et al., 2020). So far, the use of high-fishmeal (FM) diets containing high levels of HI meal up to 40% has been reported to not influence (Renna et al., 2017; Cappellozza et al., 2019; Cardinaletti et al., 2019) or worsen (St-Hilaire et al., 2007; Sealey et al., 2011; Dumas et al., 2018) the growth performance of rainbow trout (*Oncorhynchus mykiss*), with some authors also reporting unaffected (Renna et al., 2017) or reduced (Dumas et al., 2018; Cardinaletti et al., 2019) length of the intestinal villi. In parallel, the gut mucin production has been described as unaltered (Elia et al., 2018), while positive effects on the intestinal microbiota (i.e, increased microbial diversity, selection of potentially beneficial bacteria, and reduction of potential pathogens) has been identified in HI-fed fish (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019). However, the potential of using HI-based products in low-FM diets – which contain plant-derived proteins as additional protein sources – has recently started being explored at low inclusion levels only (3–15%; Caimi et al., 2021). Furthermore, no data about gut microbiota modulation in rainbow trout fed HI-based low-FM diets are available yet.

Therefore, the present study aims to evaluate the effects of including increasing levels of a partially defatted HI meal in low-FM diets for rainbow trout as partial or total replacement of FM. In particular, the attention was herein focused on the fish growth performance, nutrient digestibility coefficients, selected gut health traits, and health status indices.

2. Materials and methods

The present experiment (consisting of both a digestibility and a growth trial) was conducted at the Experimental Facility of the Department of Agricultural, Forest, and Food Sciences (DISAFA) of the University of Turin (Italy). The experimental protocol was predisposed in respect of the guidelines of the European and Italian regulations on the care and use of experimental animals (European directive 86 609/EEC). The experimental protocol was approved by the Ethical Committee of the University of Turin (protocol no 143811).

2.1. Insect meal preparation

The HI larvae were fed GMP+ certified feed of vegetal origin. Larvae were harvested, washed in cold water, slaughtered by direct mincing, pasteurized, separated by centrifugal force, and furtherly processed to obtain partially defatted meal and fat. Partially

defatted HI meal used in the present study had a shelf life of 6 months and was stored according to supplier instructions (20 °C in a cool and dry place). Processing of partially defatted meal into feed was performed within one month since production.

2.2. Experimental diets

Two diets containing FM (206 g/kg as fed; HI0) or a partially defatted HI meal produced in the experimental facility of a Dutch insect producer (Protix BV, Dongen, The Netherlands – 320 g/kg as fed; HI100) in substitution of 100% of FM were formulated by Research Diet Services BV (Utrecht, The Netherlands) and DISAFA. For nutrient digestibility evaluation, 10 g/kg as fed of Diamol (an acid insoluble ash) was added as inert marker. The two diets were formulated to be isonitrogenous, isolipidic, and isoenergetic. After that, two additional experimental diets were prepared by mixing: 1) 750 g/kg as fed of HI0 and 250 g/kg as fed of HI100 (HI25), and 2) 500 g/kg as fed of HI0 and 500 g/kg as fed of HI100 (HI50). The control diet (HI0) was formulated to mimic a commercial (low-FM) diet for rainbow trout, while the four experimental diets contained increasing levels of HI meal in substitution of 0% (HI0), 25% (HI25), 50% (HI50) and 100% (HI100) of FM (corresponding to 0, 80, 160 and 320 g/kg as fed of HI meal, respectively). The four diets (shown in Table 1) were prepared as extruded feed by Research Diet Services BV and shipped to the Experimental Facility of DISAFA. Feed was extruded using a Clextral BC45 twin-screw extruder with electrical barrel heating (Clextral, Firminy, France). The diets were stored at 0–4 °C and 85–90% RH in dark room before feeding to the fish.

2.3. Chemical analyses of feed

Feed samples were ground using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland) and analysed for dry matter (DM), crude protein (CP), acid detergent fiber (ADF) and crude ash contents (AOAC International, 2000). Feed samples were also analysed for ether extract (EE; AOAC International, 2003), neutral detergent fiber (NDF; Van Soest et al., 1991), and fatty acid (FA) profile (Renna et al., 2017). An adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany) allowed determining the GE content. All the chemical analyses of the feeds were performed in triplicate. The proximate composition and the FA profile of the experimental diets are shown in Tables 1 and 2, respectively. Feed were also sampled for the microbiota assessment (please, see "*DNA extraction and 16 S rRNA amplicon target sequencing*" subsection).

Table 1
Feed ingredients and proximate composition of the experimental diets.

	HI meal	HI0	HI25	HI50	HI100
Ingredients, g/kg as fed					
Fish meal		206	154.50	103	0
Soybean protein concentrate		150	150	150	150
Wheat gluten meal		100	100	100	100
Corn gluten meal		70	70	70	70
Soybean meal		40	40	40	40
Wheat meal		240.50	218.23	195.95	151.40
HI meal		0	80	160	320
Fish oil		50	50	50	50
Soybean oil		123.50	111.38	99.25	75
Vit. min. premix (1%)		10	10	10	10
DL methionine		0	0.28	0.55	1.10
L-lysine HCL		0	0.60	1.20	2.40
Diamol		10	10	10	10
Lime fine		0	1.62	3.25	6.50
Monocalcium phosphate		0	2	4	8
Salt		0	1.63	3.25	5
Magnesium oxide		0	0.15	0.30	0.60
Proximate composition[a]					
DM, g/kg	962.4	939.0	941.6	944.2	949.4
CP, g/kg DM[b]	517.1	443.5	445.6	447.8	452.0
EE, g/kg DM	204.3	189.0	187.8	186.5	184.0
Ash, g/kg DM	56.5	67.6	67.8	68.1	68.5
NDF, g/kg DM	N.A.	208.3	181.1	153.9	99.5
ADF, g/kg DM	N.A.	15.7	21.7	27.6	39.5
NFE, g/kg DM[c]	N.A.	238.9	240.4	241.9	244.9
GE, MJ/Kg[d]	22.04	21.74	21.85	21.57	21.56

Abbreviations: HI0, control diet; HI25, 25% of FM replaced by Hermetia illucens meal; HI50, 50% of FM replaced by Hermetia illucens meal; HI100, 100% of FM replaced by Hermetia illucens meal; DM, dry matter; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; ADFn, acid detergent fiber nitrogen; NDF, neutral detergent fiber; NFE, nitrogen-free extract.

[a] Values are reported as mean of duplicate analyses;

[b] Conversion factors of 5.62 for the HI meal (Janssen et al., 2017) and 6.25 for the experimental diets;

[c] Calculated as 100 – [(100 – DM) + CP + EE + Ash);

[d] Determined by calorimetric bomb.

2.4. *Digestibility trial*

A total of 216 trout (purchased from a private fish hatchery ["Troticoltura Bassignana", Cuneo, Italy], with a weight of 160.25 ± 8.24 g) were distributed into twelve 250-L cylindroconical tanks (3 replicate tanks/diet, 18 fish/tank) connected to a flow-through open system where artesian well water was supplied (tank water inflow: 8 L/min; T: 13 ± 1 °C; dissolved oxygen levels: 7.6–8.7 mg/L). After 14 days of acclimatization with a commercial diet (420 g/kg as fed CP and 220 g/kg as fed EE; Skretting Italia Spa, Mozzecane, Verona, Italy), the fish were fed by hand to visual satiety twice a day (8:00 am and 3:00 pm). The ADC were measured using the indirect acid-insoluble ash method, with 1% Diamol being used as inert marker. The faeces were collected daily from each tank for four consecutive week as described by Chemello et al. (2020). The faeces were frozen (−20 °C), successively freeze-dried, and stored until chemical analyses. The ADC of DM (ADCDM), crude protein (ADCCP), ether extract (ADCEE) and gross energy (ADCGE) were calculated according to Chemello et al. (2020).

2.5. *Growth trial*

A total of 600 rainbow trout were purchased from a private fish hatchery ("Troticoltura Bassignana", Cuneo, Italy). After a four-week period of acclimation (during which the fish were fed the same commercial diet used for the digestibility trial), the rainbow trout were submitted to a light anaesthesia (MS-222; PHARMAQ Ltd., Sandleheath, UK; 60 mg/L), individually weighed (112.86 ± 8.41 g) using electronic scales (KERN PLE-N v. 2.2; KERN & Sohn GmbH, Balingen-Frommern, Germany; d: 0.1), and randomly allotted to twelve 300-L, rectangular-shaped tanks (three replicate tanks per diet, fifty fish per tank) connected to the same flow-through open

Table 2
Fatty acid (FA) composition of the experimental diets.

	HI0	HI25	HI50	HI100
Fatty acids, g/100 g DM of TFA[a]				
C10:0	0.00	5.90	12.32	14.87
C12:0	7.54	363.27	756.46	1298.35
C14:0	198.88	273.55	352.25	468.21
C15:0 iso	7.37	6.87	6.75	5.61
C15:0 anteiso	9.15	8.47	10.01	8.36
C14:1 c − C15:0	24.06	25.71	26.49	27.19
C16:0 iso	4.41	4.66	4.21	4.07
C16:0	1480.80	1570.42	1627.03	1663.50
C17:0 iso	20.61	19.82	19.09	15.73
C17:0 anteiso	19.35	17.37	20.76	18.41
C16:1 c	226.49	244.14	269.18	289.74
C17:1 c9	19.94	20.02	20.43	19.80
C18:0	486.70	491.09	476.63	443.73
C18:1 t	35.64	34.58	30.05	27.40
C18:1 c9	5186.36	5028.82	4851.09	4163.97
C18:1 c11	248.71	238.81	231.19	194.62
C18:1 c12	5.71	4.15	5.62	2.26
C18:1 c14 + t 16	18.77	13.95	15.35	9.80
C18:2 n6	6284.27	6029.43	5726.58	4698.82
C20:0	37.68	41.45	38.80	37.03
C18:3 n6	9.76	8.59	7.73	8.39
C20:1 c9	41.31	40.71	35.08	28.43
C20:1c11	308.66	303.57	282.93	245.98
C18:3 n3	245.42	259.18	271.83	280.86
C20:2 n6	72.29	69.68	66.22	56.73
C18:4 n3	75.04	75.04	66.59	56.24
C22:0	9.60	10.09	10.44	9.19
C22:1 n9	322.80	290.28	270.81	227.25
C20:3 n6	41.46	39.34	35.67	30.64
C20:4 n6	24.29	22.58	19.59	12.26
C20:5 n3	295.22	279.60	251.54	193.51
C22:5 n3	62.39	60.51	59.32	52.80
C22:6 n3	235.30	192.50	175.99	132.08
Σ SFA	2371.59	2903.64	3417.38	4061.31
Σ MUFA	6414.39	6219.03	6011.73	5209.25
Σ PUFA	7279.99	6971.50	6624.90	5475.28
Σ n3	879.80	831.12	794.35	689.89
Σ n6	6405.91	6144.52	5836.18	4787.65
Σ n6/ Σ n3	7.28	7.39	7.35	6.94
TFA	16,065.97	16,094.16	16,054.02	14,745.84

Abbreviations: HI0, control diet; HI25, 25% of FM replaced by Hermetia illucens meal; HI50, 50% of FM replaced by Hermetia illucens meal; HI100, 100% of FM replaced by Hermetia illucens meal; c, cis; t, trans; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids. a Renna et al. (2014).

water system of the digestibility trial. The fish were fed 1.4% of the tank biomass for the first 123 days of trial, while the feeding rate was reduced to 1.1% for the remaining 20 days. In particular, the fish were fed by hand, twice a day (08:00 and 15:00) and six days per week. Feed intake was checked at each administration, and feed distribution was immediately interrupted if fish stopped eating. In order to choose the optimal daily feeding rate, all the biomass tanks were weighed every two weeks. Mortality was daily checked. The experimental trial lasted 133 days.

2.6. Growth performance

At the end of the growth trial, the fish were left unfed for one day, submitted to a light anesthesia and individually weighed. The following performance parameters were calculated according to Renna et al. (2017): survival, individual weight gain (iWG), feed conversion ratio (FCR), protein efficiency ratio (PER), and specific growth rate (SGR).

2.7. Condition factor and somatic indices

At the end of the growth trial, 21 fish per diet (7 fish/tank) were killed by over anaesthesia (500 mg/L) after being individually weighted. The Fulton's condition factor (K), the carcass yield (CY), the hepatosomatic index (HSI), the viscerosomatic index (VSI), and the coefficient of fatness (CF) were calculated according to Renna et al. (2017).

2.8. Sampling and processing

At the end of the growth trial, 12 fish per dietary treatment (4 fish per tank) were also killed by over anaesthesia and submitted to morphometric and histopathological investigations. Anterior (the tract immediately after the pyloric caeca) and posterior (the tract 1 cm before the anus) gut segment samples (approximately 2 cm in length) were excised, flushed with 0.9% saline to remove all the content, and fixed in 10% buffered formalin solution for morphometric investigations, while liver, spleen and stomach were sampled for histopathological examination. All the tissues were processed according to Chemello et al. (2021). The posterior gut content was also collected into sterile plastic tubes after appropriate squeezing, cooled at 4 °C (for a period not longer than 2 h), and frozen at − 80 °C until the extraction of the DNA.

2.9. Histomorphological investigations

Morphometric analysis of the gut was performed following the procedures described in details by Chemello et al. (2021). Briefly, the morphometric measurements of villus height (Vh, from the villus tip to submucosa) were performed by Image–Pro Plus 6.0 software (Media Cybernetics Inc., Bethesda, Rockville, MD, USA) on 10 well-oriented and not damaged villi (Renna et al., 2017). The observed histopathological findings were scored in all the organs according to Elia et al. (2018), while gut histopathological findings were characterized following the semi-quantitative scoring system proposed by Biasato et al. (2019).

2.10. DNA extraction and 16 S rRNA amplicon target sequencing

The total genomic DNA (gDNA) was extracted from the posterior gut content (0.25 mg) by using the Qiagen power microbiome kit (Milan, Italy) following manufactured instructions. The RNA was digested by adding 0.1 U of RNAse (Promega, Milan, Italy), and thengDNA was quantified by using the Qubit assay and diluted to 5 ng/μL. The V3-V4 region of the 16 S rRNA gene was amplified by using the following primers (Klindworth et al., 2013): 16 SF (5'-CCTACGGGNGGCWGCAG-3') and 16 SR (5'-GACTACHVGGG-TATCTAATCC-3'). The Illumina overhang adapter and PCR conditions were chosen according to the standard Illumina 16 S Metagenomic Sequencing Library Preparation. Amplicons were then purified by using the Kapa pure beads (Roche, Milan, Italy) and tagged by using the Nextera XT dual index according to the manufactory instruction. Amplicons were then quantified by using the Qubit assay, normalized at the same concentration, and pooled. Sequencing was performed on a MiSeq platform (Illumina) using the MiSeq v2 reagent cartridge in paired end mode (2x250pb).

2.11. Bioinformatics and statistical analysis

The experimental unit was the tank for growth performance and nutrient digestibility, and the fish for somatic indices, histomorphological findings and 16 S rRNA sequences.

After sequencing raw reads were imported in QIIME for denoising (>Q20) and chimera filtering step (Caporaso et al., 2010). The Operational Taxonomic Units (OTUs) were then picked at 97% of similarity and used for taxonomic assignment by using the RDP classifier against the greengenes database. The OTUs table was then build, singleton excluded and rarefied at the lowest number of sequencing per samples. The OTUs table was then filtered at > 0.2% in at least five samples. The statistical analysis of 16S rRNA sequences was performed using R software. Alpha diversity was calculated using the vegan package of R (Dixon, 2003), and all the diversity indices were compared among the experimental diets by using the function *dunn.test* of R (Kruskal-Wallis test, p-value adjustment methods Benjamini-Hochberg). The OTUs table was then used to perform Anosim statistical test and analyze the beta diversity, with Pairwise Kruskal-Wallis tests allowing the identification of significant differences in OTUs abundance according to the dietary HI meal inclusion. P-values were adjusted for multiple testing and a false discovery rate (FDR) < 0.05 considered as significant.

The statistical analysis of growth performance, nutrient digestibility, somatic indices and histomorphological findings was performed using IBM SPSS Statistics v. 27.0 (IBM, Armonk, NY, USA). One-way ANOVA was used to compare growth performance, nutrient digestibility and somatic indices data among the dietary treatments. The Shapiro–Wilk test assessed the normality or non-normality distribution of the dependent variables. The assumption of equal variances was assessed by Levene's homogeneity of variance test, and, if such an assumption did not hold, the Brown-Forsythe statistic was performed. Tukey's and Tamhane's T2 tests were chosen as post-hoc tests in the cases of equal variances assumed or not assumed, respectively. The morphometric indices were analysed by fitting a general linear model that allowed the morphometric indices (Vh) to depend on three fixed factors (diet, intestinal segment, and the corresponding interaction). The interactions between the levels of the fixed factors were evaluated by pairwise contrasts. Histopathological scores were analysed by Chi-square test. The results obtained from normally distributed data were expressed as mean (growth performance, nutrient digestibility and somatic indices) or least square mean (morphometric indices) and pooled standard error of the mean (SEM), while those obtained from not normally distributed data (histopathological findings) as n (%). P values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Chemical analyses of the feed

The experimental diets were all comparable in terms of macronutrients, as the differences between the lowest and the highest CP, EE and GE contents were lower than 5% (1.92%, 2.65%, and 1.33%, respectively; Table 1). As far as the FA profile is concerned (Table 2), the lauric (C12:0), myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1c9) and linoleic (C18:2 n6) acids were the most represented FA in all the experimental diets. In particular, the lauric, myristic, and palmitic acids increased with increasing HI meal inclusion levels, while the oleic and linoleic acids displayed the opposite trend (Table 2). Subsequently, the total saturated fatty acids (SFA) increased on increasing the insect meal, whereas the total monounsaturated and polyunsaturated fatty acids (MUFA and PUFA, respectively) decreased (Table 2). The decrease in the PUFA was determined by the decrease in the arachidonic (C20:4 n6), eicosapentaenoic (EPA, C20:5 n3), docosapentaenoic (DPA, C22:5 n3) and docosahexaenoic (DHA, C22:6 n3) acids, thus furtherly explaining the progressive reduction in the n3 and n6 FA (and the n6/n3 as well) identified in the experimental diets (Table 2).

3.2. Digestibility trial

The ADCs of the nutrients observed in the rainbow trout were not significantly influenced by dietary HI meal inclusion ($P > 0.05$, Table 3).

3.3. Growth trial

3.3.1. Growth performance

Growth performance of the rainbow trout are summarized in Table 4. The fish did not refuse the experimental diets, with all the supplied feed being daily consumed during the experimental trial. Survival was high for all the dietary treatments (range: 95.33–97.33), being also not influenced by HI meal utilization ($P > 0.05$, Table 4). Similarly, all the other growth parameters were not affected by insect meal inclusion ($P > 0.05$, Table 4).

3.3.2. Condition factor and somatic indices

The inclusion of HI meal in rainbow trout diets did not influence either the condition factor or the somatic indices of the fish ($P > 0.05$, Table 5).

3.4. Histomorphological investigations

Data regarding the morphometric measurements of the Vh in the anterior and posterior gut are reported in Table 6. The Vh was not influenced by the diet and the interaction between the diet and the intestinal segment ($P > 0.05$, Table 6), but it only depended on the intestinal segment ($P < 0.001$, Table 6). Independently of the dietary HI meal inclusion, the Vh progressively increased from the

Table 3
Apparent digestibility coefficients of dry matter, protein, ether extract and gross energy of the rainbow trout (n = 4).

	HI0	HI25	HI50	HI100	SEM	P-value
ADC DM (%)	84.54	87.50	84.71	84.67	0.67	0.346
ADC CP (%)	95.07	95.85	94.44	94.32	0.25	0.086
ADC EE (%)	98.43	98.73	98.37	98.33	0.08	0.298
ADC GE (%)	92.26	93.10	91.25	90.84	0.41	0.192

Abbreviations: HI0, control diet; HI25, 25% of FM replaced by Hermetia illucens meal; HI50, 50% of FM replaced by Hermetia illucens meal; HI100, 100% of FM replaced by Hermetia illucens meal; SEM, standard error of the mean; P, probability; ADC, apparent digestibility coefficient; DM, dry matter; CP, crude protein; EE, ether extract; GE, gross energy.

Table 4
Survival and growth performance of the rainbow trout (n = 3).

	HI0	HI25	HI50	HI100	SEM	P-value
Survival (%)	96.00	95.33	97.33	96.67	0.48	0.557
IBW (g)	112.73	113.13	112.70	112.93	0.08	0.142
FBW (g)	467.53	463.60	469.37	474.70	3.33	0.756
iWG (g)	354.87	350.49	356.66	361.77	3.33	0.748
FCR	1.72	1.73	1.77	1.75	0.02	0.856
PER	1.33	1.32	1.26	1.25	0.02	0.299
SGR (% day^{-1})	0.90	0.90	0.89	0.91	0.01	0.762

Abbreviations: HI0, control diet; HI25, 25% of FM replaced by Hermetia illucens meal; HI50, 50% of FM replaced by Hermetia illucens meal; HI100, 100% of FM replaced by Hermetia illucens meal; SEM, standard error of the mean; P, probability; iIBW, individual initial body weight; iFBW, individual final body weight; iWG, individual weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio.

Table 5
Condition factor and somatic indices of the rainbow trout (n = 21).

	HI0	HI25	HI50	HI100	SEM	p-value
K	1.19	1.12	1.16	1.16	0.02	0.548
CY	89.98	88.72	89.17	89.37	0.26	0.392
HSI	1.12	1.08	1.07	1.08	0.03	0.938
VSI	8.42	8.47	8.16	7.88	0.15	0.488
CF	3.52	3.86	3.62	3.26	0.14	0.465

Abbreviations: HI0, control diet; HI25, 25% of FM replaced by Hermetia illucens meal; HI50, 50% of FM replaced by Hermetia illucens meal; HI100, 100% of FM replaced by Hermetia illucens meal; SEM, standard error of the mean; P, probability; K, condition factor; CY, carcass yield; HIS, hepatosomatic index; VSI, viscerosomatic index; CF, coefficient of fatness.

anterior to the posterior gut ($P < 0.001$, Table 6).

The histopathological alterations observed in liver, spleen, stomach, anterior and posterior gut are summarized in Table 7. In liver, absent to mild, focal to multifocal lymphoplasmacytic infiltrates, as well as absent to moderate, multifocal to diffuse fatty changes of the hepatocytes were observed in all the dietary treatments. Mild, focal to multifocal hemosiderosis, along with moderate, focal to multifocal white pulp hyperplasia were also recorded in all the experimental groups. No signs of immune cell infiltration were observed in the stomach, except for the HI100 group (8.3%). All the fish displayed mild, focal to multifocal lymphoplasmacytic infiltrates in both the anterior and the posterior intestine. However, dietary HI meal inclusion did not influence either the severity or the distribution of the observed histopathological alterations ($P > 0.05$, Table 7).

3.5. Feed 16S rRNA amplicon target sequencing

The feed samples were overall characterized by a simple microbiota, with Firmicutes, Cyanobacteria and Proteobacteria representing the main bacterial phyla, and *Lactobacillus*, *Listeria*, *Leuconostoc*, *Streptococcus* and *Photobacterium* the most abundant genera (Fig. 1). Increasing levels of HI meal in the feeds determined a progressive, numerical increase in the relative abundance of Firmicutes and Actinobacteria phyla, whereas Proteobacteria displayed a gradual, numerical reduction (Fig. 1A). Furthermore, the relative abundance of *Lactobacillus* and *Listeria* numerically decreased with increasing percentages of HI meal, while *Staphylococcus*, *Enterococcus*, *Oceanobacillus* and *Actinomyces* showed the opposite trend (Fig. 1B).

3.6. Posterior gut 16 S rRNA amplicon target sequencing

After sequencing and quality filtering, 895,348 reads were used for the downstream analysis (with a median value of 18,371 ± 10,395 reads/sample). The rarefaction analysis and the estimated sample coverage indicated that there was a satisfactory coverage of all the samples (ESC median value of 96%). The alpha diversity analysis also revealed a significant increase in the Chao1 index of the posterior gut microbiota from the HI-fed rainbow trout, whereas the Shannon index displayed the opposite trend ($P < 0.05$, Fig. 2). By

Table 6
Intestinal morphometric indices of the rainbow trout (n = 12).

	Diet (D)				Intestinal segment (IS)		SEM		P-value		
	HI0	HI25	HI50	HI100	Anterior	Posterior	D	IS	D	IS	D x IS
Vh (mm)	0.87	0.83	0.80	0.79	0.68^{a}	0.96^{b}	0.33	0.02	0.392	< 0.001	0.982

Abbreviations: HI0, control diet; HI25, 25% of FM replaced by Hermetia illucens meal; HI50, 50% of FM replaced by Hermetia illucens meal; HI100, 100% of FM replaced by Hermetia illucens meal; SEM, standard error of the mean; P, probability; Vh, villus height.
Means with different superscript letters (a, b) indicate significant differences.

Table 7
Histopathological alterations of the rainbow trout (n = 12).

	Dietary treatments				
Variables	HI0	HI25	HI50	HI100	P-value
Liver *n (%)*					
Inflammation					0.110
Absent	12 (100)	12 (100)	9 (75)	10 (83.3)	
Mild	0 (0)	0 (0)	3 (25)	2 (16.7)	
Degeneration					0.088
Absent	0 (0)	1 (8.3)	5 (42)	3 (25)	
Mild	5 (41.7)	7 (58.3)	5 (42)	8 (66.7)	
Moderate	6 (50)	4 (33.4)	2 (16.7)	0 (0)	
Severe	1 (8.3)	0 (0)	0 (0)	1 (8.3)	
Spleen *n (%)*					
White pulp hyperplasia					0.495
Absent	9 (81.8)	10 (90.9)	11 (91.7)	12 (100)	
Mild	2 (18.2)	1 (9.1)	1 (8.3)	0 (0)	
Hemosiderosis					0.347
Absent	3 (27.3)	6 (54.5)	4 (33.3)	7 (58.3)	
Mild	8 (72.7)	5 (45.5)	8 (66.7)	5 (41.7)	
Stomach inflammation *n (%)*					0.395
Absent	12 (100)	12(100)	12 (100)	10 (83.4)	
Mild	0 (0)	0 (0)	0 (0)	1(8.3)	
Anterior gut inflammation *n (%)*					1.00
Absent	9 (75)	9 (75)	9 (75)	9 (75)	
Mild	3 (25)	3 (25)	3 (25)	3 (25)	
Posterior gut inflammation *n (%)*					0.681
Absent	11 (91.7)	9 (75)	9 (75)	10 (83.3)	
Mild	1(8.3)	3 (25)	3 (25)	2 (16.7)	

Abbreviations: HI0, control diet; HI25, 25% of FM replaced by Hermetia illucens meal; HI50, 50% of FM replaced by Hermetia illucens meal; HI100, 100% of FM replaced by Hermetia illucens meal; SEM, standard error of the mean; P, probability.

plotting the Principal Component Analysis (PCA), a clear separation between the fish fed the control and the HI-based diets was also observed, with a higher diversity being furtherly identified in the posterior gut microbiota from the HI25 rainbow trout when compared to the HI50 and HI100 groups ($P < 0.001$, Fig. 3).

The characterization of the posterior gut microbiota of the rainbow trout overall revealed Firmicutes, Actinobacteria and Proteobacteria as predominant phyla (Fig. 4A), while *Staphylococcus, Lactobacillus, Enterococcus, Oceanobacillus, Actinomyces, Streptococcus* and *Weissella* resulted to be the most abundant genera (Fig. 4B). At phylum level (Fig. 5), the HI25 and the HI50 fish showed increased relative abundance of Actinobacteria in comparison with the HI0 group (FDR < 0.05). On the contrary, Bacteroidetes phylum was significantly less abundant in the rainbow trout fed the HI25 and the HI50 diets when compared to the HI0 one (FDR < 0.05). As far as genus level is concerned (Fig. 6), the HI-fed fish showed a significant increase in the relative abundance of *Actinomyces, Bacillus, Enterococcus, Oceanobacillus,* and *Staphylococcus* (FDR < 0.05). Differently, the relative abundance of *Clostridium, Campylobacter, Listeria, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus,* unclassified members (U.m.) of Peptostreptococceae, *Vagococcus,* and *Weissella* genera was significantly decreased in the rainbow trout fed the HI-based diets in comparison with the HI0 group. No changes related to the different HI meal inclusion levels were, however, identified for both the phyla and the genera (FDR > 0.05).

4. Discussion

4.1. Digestibility trial

The apparent digestibility of the nutrients and the energy of the HI-based diets was analogous to that recorded for the C diet, as already underlined by previous research (Renna et al., 2017; Caimi et al., 2021). This is indicative of a good, proper nutrient availability, which reasonably explains the unaffected growth performance highlighted in the HI-fed fish.

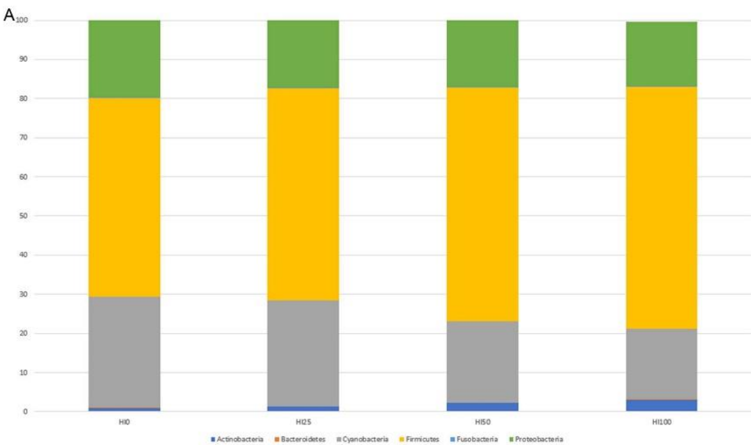
4.2. Growth trial

4.2.1. Growth performance

The growth performance of the rainbow trout of the present study were not affected by dietary HI meal inclusion, thus reflecting the unaltered nutrient digestibility coefficients, and being already underlined by previous research (Renna et al., 2017; Cappellozza et al., 2019; Cardinaletti et al., 2019; Caimi et al., 2021).

4.3. Condition factor and somatic indices

Both the condition factor and the somatic indices of the rainbow trout of the present study were not significantly influenced by



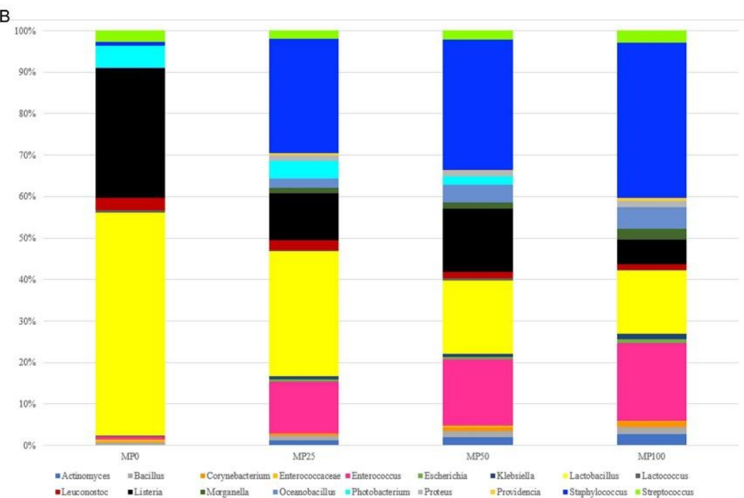


Fig. 1. Relative abundance of the main bacterial phyla (**A**) and genera (**B**) in samples of commercial feeds containing low content of fishmeal (HI0), *Hermetia illucens* meal as replacement of 25% of fishmeal (HI50), *Hermetia illucens* meal as replacement of 50% of fishmeal (HI50), and *Hermetia illucens* meal as replacement of 100% of fishmeal (HI100).

dietary HI meal inclusion. This is in agreement with previous research studies about HI meal utilization in rainbow trout, which also reported analogous K (Renna et al., 2017; Cardinaletti et al., 2019), HSI (Sealey et al., 2011) and VSI (Bruni et al., 2018) values. The so-obtained results confirm that HI meal utilization allows the preservation of a good physiological state in fish, without determining

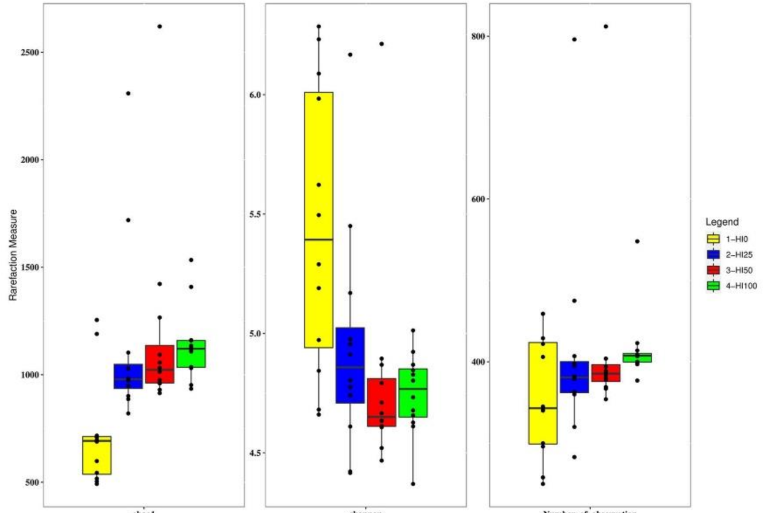


Fig. 2. Bacterial community alpha diversity in posterior gut samples of rainbow trout fed control (HI10), *Hermetia illucens* meal as replacement of 25% of fishmeal (HI50), *Hermetia illucens* meal as replacement of 50% of fishmeal (HI50), and *Hermetia illucens* meal as replacement of 100% of fishmeal (HI100) diets. Box plots with different superscript letters (a, b) indicate significant differences among the treatments (P < 0.05).

metabolic problems or liver and gastrointestinal diseases (as analogously confirmed by the histopathological examination).

4.4. *Histomorphological features*

Dietary HI meal inclusion did not significantly affect the gut morphology of the rainbow trout of the current research, as already reported by Renna et al. (2017). This is in agreement with the unaffected growth performance herein observed in the HI-fed fish, thus suggesting no negative repercussions on either the digestion or the absorption of the nutrients by the intestine. Independently of HI utilization, the posterior intestine of the rainbow trout of the present study showed higher villi than the anterior gut. This scenario disagrees with that one underlined by Khojasteh et al. (2009), which reported a progressive decrease in villi length from the anterior to the posterior intestine. However, the concomitant identification of short and long villi in both the gut segments – as well as the villi length changes throughout the fish cycle – has recently been reported in rainbow trout (Verdile et al., 2020), thus making further investigations needed.

The histopathological findings observed in the fish of the current research were also not significantly influenced by HI meal utilization, thus being in agreement with previous research studies (Elia et al., 2018), and confirming no negative effects of HI on fish health. The fatty and inflammatory changes in liver and gastrointestinal tract, respectively, are the common result of the high-energy diet administered to salmonids, while the spleen reactivity appears to be aspecific. Furthermore, the histopathological alterations were highlighted in both the control- and the HI-fed fish, also resulting to be predominantly mild to moderate (and, in turn, of negligible relevance).

4.5. *Feed and gut microbiota*

Firmicutes, Cyanobacteria and Proteobacteria phyla dominated the microbiota of the feed used in the present study. This is partially in agreement with Terova et al. (2019), which identified a predominance of Firmicutes, Proteobacteria and Actinobacteria in FM-based diets for rainbow trout. However, the detection of high percentages of Cyanobacteria represents an unexpected finding. Cyanobacteria has recently been found in the gut microbiota of marine (Salas-Leiva et al., 2020) and freshwater (Jiang et al., 2019; Zeng et al., 2020) species, being also one of the most abundant prokaryotes in sea (Korlević et al., 2016; Quéméneur et al., 2020) and anthropogenic-induced eutrophied freshwaters (Zhang et al., 2021). Considering that the biomass which supplies the FM industry is

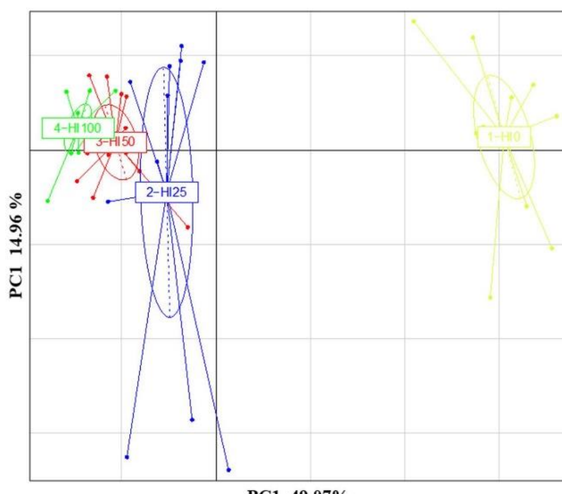


Fig. 3. Bacterial community composition (PCA plots) in posterior gut samples of rainbow trout fed control (HI10), *Hermetia illucens* meal as replacement of 25% of fishmeal (HI50), *Hermetia illucens* meal as replacement of 50% of fishmeal (HI50), and *Hermetia illucens* meal as replacement of 100% of fishmeal (HI100) diets.

mainly composed of small pelagic species (Péron et al., 2010), it seems reasonable that the feed microbiota herein characterized reflect the gut microbiota of the fish species (and their rearing environment as well) used to produce the FM. The identification of high relative abundances of Firmicutes and Proteobacteria – which are two of the dominant bacterial phyla of the fish gut microbiota (Butt and Volkoff, 2019) – further supports such hypothesis. A similar consideration can also be made for the most represented bacterial genera detected in feed microbiota, as *Lactobacillus* (Tarnecki et al., 2017; Huyben et al., 2020; Yu et al., 2021), *Leuconostoc*, *Streptococcus* (Tarnecki et al., 2017) and *Photobacterium* (Huyben et al., 2020) constitute the core microbiota of several marine species, with the latter OTU being particularly characteristic of piscivores such the pelagic species (Huang et al., 2020). Differently, the detection of high percentages of *Listeria* may rise worrying concerns in terms of food safety, as some species (especially *L. monocytogenes*) are involved in foodborne outbreaks of listeriosis (Buchanan et al., 2017). Since the consumption of raw and smoked seafood is one of the most common predisposing factor to develop such disease and *Listeria* has frequently been isolated in marine finfish (Basha et al., 2019), the fish species herein used to produce the FM could have potentially carried *Listeria* to the feeds.

The HI-based diets used in the current research were characterized by a progressive increase in the relative abundance of Firmicutes and Actinobacteria phyla, whereas Proteobacteria displayed a gradual reduction. This is in agreement with Terova et al. (2019), which described the same scenario in feeds containing increasing levels of HI prepupa meal as FM replacement. This represents the logical consequence of substituting the FM (which is obtained by carnivorous fish) with the insect meal (which is obtained by larvae reared on vegetable substrates). Indeed, plant ingredients in the diet are commonly associated with a higher Firmicutes:Proteobacteria ratio when compared to animal protein-based diet, which, on the contrary, stimulates the proliferation of Proteobacteria (Rimoldi et al., 2018). A clear increase in the relative abundance of *Staphylococcus*, *Enterococcus*, *Oceanobacillus* and *Actinomyces* was also identified in the HI-based diets, thus partially agreeing with the findings reported by Terova et al. (2019). The detection of increasing percentages of *Oceanobacillus* represents, however, a novel, difficult-to-explain result, as this taxon has been reported to dominate the gut microbiota of healthy shrimp, crab and clam (Sun et al., 2019). Furthermore, despite Rimoldi et al. (2021) having recently discovered *Oceanobacillus* in HI-based feed only, its relationship with insects remains to be fully elucidated. High amounts of *Lactobacillus* in diets containing HI meal are also common (Terova et al., 2019; Rimoldi et al., 2021), while the HI-based feeds used in the present study displayed a progressive reduction of this genus. This finding – as well as the decrease of *Listeria* – is reasonably related to the FM replacement by insect meal, as these OTUs are herein hypothesized to depend on the fish species used to produce the FM.

Dietary HI meal inclusion increased the gut microbial richness in the fish of the current research, but, at the same time, reduced its diversity. This partially contrasts with the majority of the previous findings in rainbow trout, which identified unaffected or higher Chao1 and Shannon indices in the HI-fed fish when compared to those fed the control diet (Bruni et al., 2018; Huyben et al., 2019;

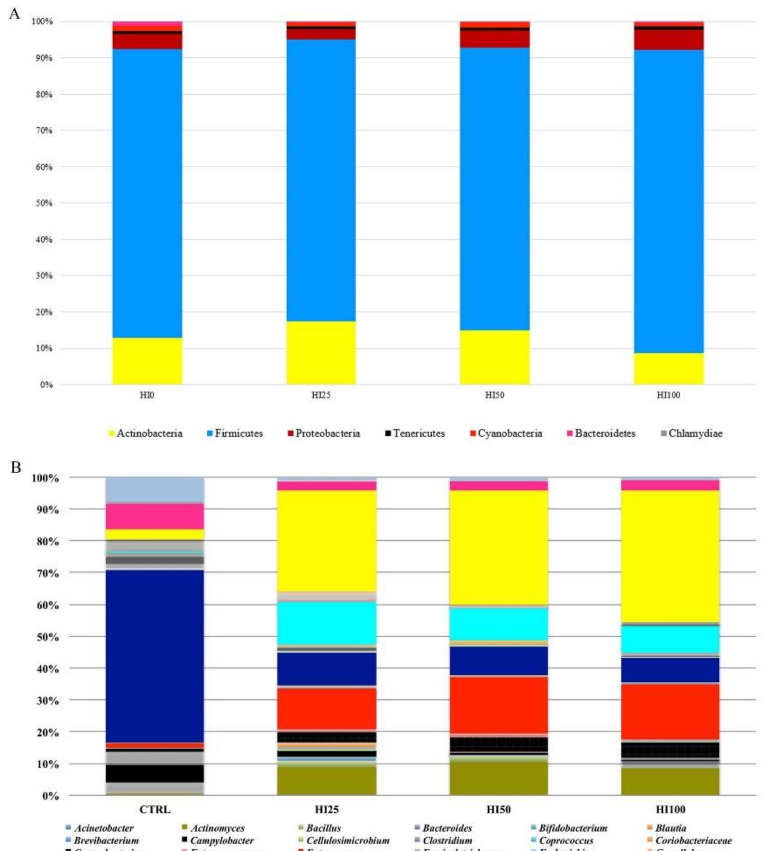


Fig. 4. Relative abundance of the main bacterial phyla (A) and genera (B) in posterior gut samples of rainbow trout fed control (HI10), *Hermetia illucens* meal as replacement of 25% of fishmeal (HI50), *Hermetia illucens* meal as replacement of 50% of fishmeal (HI50), and *Hermetia illucens* meal as replacement of 100% of fishmeal (HI100) diets.

Rimoldi et al., 2019, 2021; Terova et al., 2019). This represents a challenging scenario, as reduced bacterial diversity may determine less competition for incoming pathogens, thus favouring their colonization of the gastrointestinal tract of fish and the development of several diseases frequently related to several diseases (Terova et al., 2019). However, the rainbow trout fed the HI-based diets of the present study remained healthy during the feeding trial, also showing no remarkable histopathological lesions.

Firmicutes, Actinobacteria and Proteobacteria represented the major phyla in either the control- or the HI-fed fish of the current research. These findings are in overall agreement with the previous studies carried out in rainbow trout (Desai et al., 2012; Wong et al.,

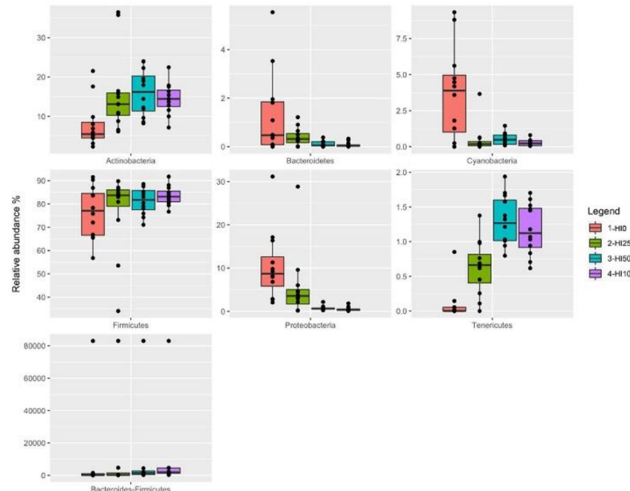


Fig. 5. Relative abundance at phylum level of differentially abundant OTUs in in posterior gut samples of rainbow trout fed control (HI10), *Hermetia illucens* meal as replacement of 25% of fishmeal (HI50), *Hermetia illucens* meal as replacement of 50% of fishmeal (HI50), and *Hermetia illucens* meal as replacement of 100% of fishmeal (HI100) diets. Box plots with different superscript letters (a, b) indicate significant differences among the treatments (FDR < 0.05).

2013; Ingerslev et al., 2014; Bruni et al., 2018; Rimoldi et al., 2018; Huyben et al., 2019; Terova et al., 2019; Pelusio et al., 2020). As far as the genera are concerned, *Staphylococcus*, *Lactobacillus* and *Streptococcus* mainly colonized the posterior gut microbiota of the fish fed both the control and the HI-based diets in the present study. *Lactobacillus* (Wong et al., 2013; Ingerslev et al., 2014; Rimoldi et al., 2018; Huyben et al., 2019; Terova et al., 2019; Pelusio et al., 2020), *Streptococcus* (Ingerslev et al., 2014; Rimoldi et al., 2018; Pelusio et al., 2020) and *Staphylococcus* (Bruni et al., 2018; Terova et al., 2019) have previously been reported as main bacterial genera in the cecal microbiota of rainbow trout, thus analogously confirming the identification of a physiological bacterial community.

In the current research, the utilization of HI meal at 25% and 50% inclusion levels determined higher relative abundance of Actinobacteria phylum in the fish posterior gut microbiota when compared to the HI0 group. A significant increase in Actinobacteria has also previously been reported in HI-fed rainbow trout (Huyben et al., 2019; Terova et al., 2019), as well as the increment in Firmicutes (Bruni et al., 2018; Huyben et al., 2019; Terova et al., 2019) and the reduction of Proteobacteria (Huyben et al., 2019; Terova et al., 2019). On one hand, the increase in Actinobacteria herein observed partially reflects the high relative abundance of this bacterial phylum detected in the HI-based diets; on the other, some genera belonging to Actinobacteria (such as *Actinomyces)* are often identified as chitin degraders (Beier and Bertilsson, 2013), thus partially explaining its high abundance in the HI-fed rainbow trout. Despite Firmicutes and Proteobacteria percentages being similar among the experimental treatments, the HI25 and the HI50 fish of the present study also displayed lower relative abundance of Bacteroidetes in their posterior gut microbiota in comparison with the HI0 group. Bacteroidetes members are well-known to be involved in the fermentation of dietary non-starch polysaccharides (NSP; den Besten et al., 2013). Since the HI-based diets were characterized by a progressive reduction of wheat meal content (which has considerable quantity of NSP), the decrease in Bacteroidetes may represent a reasonable consequence. Chitin is another NSP, but the chitinolytic bacteria mainly belong to Firmicutes (Cody, 1989) and Actinobacteria (Beier and Bertilsson, 2013) phyla, thus furtherly explaining the reduction of Bacteroidetes herein observed.

Actinomyces, *Bacillus*, *Enterococcus*, *Oceanobacillus*, and *Staphylococcus* resulted to be enriched in the posterior gut microbiota of the HI-fed rainbow trout of the current research. On the one hand, this partially reflects the microbiota of the HI-based feeds (characterized by high percentages of *Actinomyces*, *Enterococcus*, *Oceanobacillus*, and *Staphylococcus*); on the other, these changes can be attributable to chitin. Indeed, apart from the already mentioned chitin degrading activity of *Actinomyces* (Beier and Bertilsson, 2013), many *Bacillus* species are chitinolytic (Cody, 1989). As lactic acid bacteria (LAB), *Enterococcus* is also capable of using chitin as prebiotic (Terova et al., 2019), while novel chitinolytic *Staphylococcus* species have recently been characterized (Gürkök and Görmez, 2016). In agreement with the findings herein observed, a significant increase in *Actinomyces*, *Enterococcus* (Terova et al., 2019), *Staphylococcus* (Bruni et al., 2018) and *Bacillus* (Rimoldi et al., 2021) has also been reported in rainbow trout fed diets containing HI meal. These

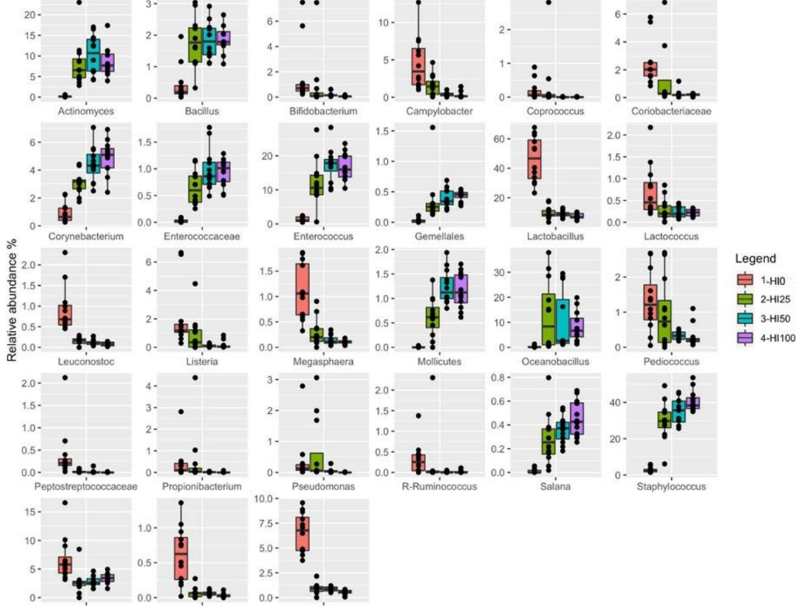


Fig. 6. Relative abundance at genus level of differentially abundant OTUs in in posterior gut samples of rainbow trout fed control (HI10), *Hermetia illucens* meal as replacement of 25% of fishmeal (HI50), *Hermetia illucens* meal as replacement of 50% of fishmeal (HI50), and *Hermetia illucens* meal as replacement of 100% of fishmeal (HI100) diets. Box plots with different superscript letters (a, b) indicate significant differences among the treatments (FDR < 0.05).

changes can be beneficial for the health status of the fish gut, as bacterial fermentation of chitin leads to short-chain fatty acids (SCFAs) production (Borrelli et al., 2017; Yu et al., 2019). Indeed, SCFAs (such as butyric, propionic and acetic acids) act as energy source, promote the proliferation of intestinal epithelial cells, exert the antimicrobial activity by lowering intestinal pH, modulate the composition of intestinal microbiota, and enhance the immune response of the fish (Li et al., 2019). In the present study, dietary HI meal inclusion also determined a significant reduction of *Clostridium*, *Campylobacter*, *Listeria*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, unclassified members (U.m.) of Peptostreptococceae, *Vagococcus,* and *Weissella* in the fish gut microbiota. The decrease in LAB such as *Lactobacillus*, *Leuconostoc* and *Pediococcus* – which have been reported to proliferate in HI-fed rainbow trout (Huyben et al., 2019; Terova et al., 2019; Rimoldi et al., 2021) – seems difficult to explain, especially because *Enterococcus* (previously described as LAB) was, however, significantly enriched. This discrepancy may be caused by the different HI meal adopted (prepupae [Terova et al., 2019] vs larvae), but the capability of insects to stimulate the growth of some LAB at the expense of others deserves future investigations. The reduction of *Clostridium* could not represent a relevant finding, since this taxon is characteristic of the intestinal microbiota from endotherms (Eckburg et al., 2005) and is involved in the degradation of the cellulolytic fibers (which are not predominant in diets for carnivorous fish) (Chapagain et al., 2019). A similar consideration can also be made for Peptostreptococcaceae family, whose members exert the generic function of utilizing proteinaceous substrates and carbohydrates (Fu et al., 2019). On the contrary, the decrease in *Weissella* may represent a potential challenging outcome, as this genus includes probiotic bacteria (Kühlwein et al., 2013) and displays antimicrobial activity against a wide range of microorganisms (Patterson et al., 2010). However, such reduction could have successfully been compensated by the chitin and the lauric acid contained in the HI meal, which have been reported to exert antimicrobial activity against both the Gram-negative (Marono et al., 2017) and the Gram-positive (Skrivanova et al., 2006) bacteria. As a reasonable consequence, the HI antimicrobial properties may have determined the decrease in *Lactococcus*, *Vagococcus*, *Campylobacter* and *Listeria*. Indeed, the reduction of *Lactococcus* and *Vagococcus* – whose distinct species have been related

to the development of a growing number of diseases (Ringø and Gatesoupe, 1998) – can be considered a positive finding, but the most remarkable HI-related outcome is represented by the decreased proliferation of *Campylobacter* and *Listeria*. Similarly, to what was already pointed out for *Listeria*, *Campylobacter* is one of the most common agents of food-borne diseases (Kreling et al., 2020), thus making their reduction particularly interesting within a food safety scenario. The reduced percentage of *Listeria* identified in the HI-based diets could also partially explain its reduction in the gut, but the difference in the corresponding relative abundances (about 7% vs 0.4%) reasonably suggests an active role of HI meal as well.

5. Conclusions

In conclusion, HI meal can be used in low-FM diets for rainbow trout up to high inclusion levels (320 g/kg as fed) without negatively affecting the growth performance, nutrient digestibility, somatic indices and histomorphological features of the animals. Therefore, considering that the low FM-diets are nowadays the most adopted fish feeds from a sustainability perspective, the possibility of including either low or high inclusion levels of HI meal without incurring in adverse outcomes represents a promising scenario. Furthermore, a positive modulation of the gut microbiota in terms of selection of SCFAs-producing bacteria and reduction of foodborne disease-causing pathogens was herein observed for the first time when rainbow trout were administered with low FM-diets containing HI meal. In the light of such positive findings, future investigations also assessing the gut metagenome and metabolome are mandatory in order to fully characterize the HI way of action in the fish gut.

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CRediT authorship contribution statement

Ilaria Biasato: conduct the experiment, sampling, statistical analysis, and writing the initial draft, **Giulia Chemello**: conduct the experiment, sampling, statistical analysis, and writing the initial draft, **Sara Bellezza Oddon**: fish feeding, sampling, and reviewing the final draft, **Ilario Ferrocino**: feed and gut microbiota analyses, and reviewing the final draft, **Christian Caimi:** fish feeding, sampling, and reviewing the final draft, **Andrea Resconi**: fish feeding, sampling, and reviewing the final draft, **Aman Paul**: feed production and reviewing the final draft, **Michel van Spankeren**: feed production and reviewing the final draft, **Maria Teresa Capucchio**: histomorphological analysis and reviewing the final draft, **Elena Colombino:** histomorphological analysis and reviewing the final draft, **Luca Cocolin:** feed and gut microbiota analyses, and reviewing the final draft, **Francesco Gai**: planning the research activity and reviewing the final draft, **Achille Schiavone**: planning the research activity and reviewing the final draft, **Laura Gasco**: coordination, funding acquisition, planning the research activity, and reviewing the final draft.

Declaration of Competing Interest

There are no competing financial, professional, or personal interests that might have influenced the presentation of the work described in this manuscript.

Data Availability

The metataxonomic sequences are available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the BioProject accession numbers PRJNA783153.

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References

AOAC International, 2000. Official Methods of Analysis of AOAC International, sixteenth ed. Association of Official Analytical Chemists, Gaithersburg.
AOAC International, 2003. Official Methods of analysis of AOAC International, sventeenth ed. Association of Official Analytical Chemists, Gaithersburg.
Basha, K.A., Kumar, N.R., Das, V., Reshmi, K., Rao, B.M., Lalitha, K.V., Joseph, T.C., 2019. Prevalence, molecular characterization, genetic heterogeneity and antimicrobial resistance of *Listeria monocytogenes* associated with fish and fishery environment in Kerala, India. Lett. Appl. Microbiol. 69, 286–293. https://doi.org/10.1111/lam.13205.
Beier, S., Bertilsson, S., 2013. Bacterial chitin degradation-mechanisms and ecophysiological strategies. Front. Microbiol. 4, 149. https://doi.org/10.3389/fmicb.2013.00149.
den Besten, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D.J., Bakker, B.M., 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J. Lipid Res. 54, 2325–2340. https://doi.org/10.1194/jlr.R036012.
Biasato, I., Renna, M., Gai, F., Dabbou, S., Meneguz, M., Perona, G., Martinez, S., Lajusticia, A.C.B., Bergagna, S., Sardi, L., Capucchio, M.T., Bressan, E., Dama, A., Schiavone, A., Gasco, L., 2019. Partially defatted black soldier fly larva meal inclusion in piglet diets: effects on the growth performance, nutrient digestibility, blood profile, gut morphology and histological features. J. Anim. Sci. Biotechnol. 10, 12. https://doi.org/10.1186/s40104-019-0325-x.

Borrelli, L., Coretti, L., Dipineto, L., Bovera, F., Menna, F., Chiariotti, L., Nizza, A., Lembo, F., Fioretti, A., 2017. Insect-based diet, a promising nutritional source, modulates gut microbiota composition and SCFAs production in laying hens. Sci. Rep. 7, 16269. https://doi.org/10.1038/s41598-017-16560-6.

Bruni, L., Pastorelli, R., Viti, C., Gasco, L., Parisi, G., 2018. 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 487, 56–63. https://doi.org/10.1016/j.aquaculture.2018.01.006.

Buchanan, R.L., Gorris, L.G.M., Hayman, M.M., Jackson, T.C., Whiting, R.C., 2017. A review of *Listeria monocytogenes*: an update on outbreaks, virulence, dose-response, ecology, and risk assessments. Food Control. 75, 1–13. https://doi.org/10.1016/j.foodcont.2016.12.016.

Butt, R.L., Volkoff, H., 2019. Gut microbiota and energy homeostasis in fish. Front. Endocrinol. (Lausanne). 10, 9. https://doi.org/10.3389/fendo.2019.00009.

Caimi, C., Gasco, L., Biasato, I., Malfatto, V., Varello, K., Prearo, M., Pastorino, P., Bona, M.C., Francese, D.F., Schiavone, A., Elia, A.C., Dörr, A.J.M., Gai, F., 2020. Could dietary black soldier fly meal inclusion affect the liver and intestinal histological traits and the oxidative stress biomarkers of Siberian sturgeon (*Acipenser baerii*) juveniles? Animals 10, 155. https://doi.org/10.3390/ani10010155.

Caimi, C., Biasato, I., Chemello, G., Bellezza Oddon, S., Lussiana, C., Malfatto, V.M., Capucchio, M.T., Colombino, E., Schiavone, A., Gai, F., Trocino, A., Brugiapaglia, A., Renna, M., Gasco, L., 2021. Dietary inclusion of a partially defatted black soldier fly (*Hermetia illucens*) larva meal in low fishmeal-based diets for rainbow trout (*Oncorhynchus mykiss*). J. Anim. Sci. Biotechnol. 12, 50. https://doi.org/10.1186/s40104-021-00575-1.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, K., Gordon, J.I., Huttley, G., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303.

Cappellozza, S., Leonardi, M.G., Savoldelli, S., Carminati, D., Rizzolo, A., Cortellino, G., Terova, G., Moretto, E., Badaile, A., Concheri, G., Saviane, A., Bruno, D., Bonelli, M., Caccia, S., Casartelli, M., Tettamanti, G., 2019. A first attempt to produce proteins from insects by means of a circular economy. Animals 9, 278. https://doi.org/10.3390/ani9050278.

Cardinaletti, G., Randazzo, B., Messina, M., Zarantoniello, M., Giorgini, E., Zimbelli, A., Bruni, L., Parisi, G., Olivotto, I., Tulli, F., 2019. Effects of graded dietary inclusion level of full-fat *Hermetia illucens* prepupae meal in practical diets for rainbow trout (*Oncorhynchus mykiss*). Animals 9, 1–15. https://doi.org/10.3390/ani9050251.

Chapagain, P., Arivett, B., Cleveland, B.M., Walker, D.M., Salem, M., 2019. Analysis of the fecal microbiota of fast- and slow-growing rainbow trout (*Oncorhynchus mykiss*). BMC Genom. 20, 788. https://doi.org/10.1186/s12864-019-6175-2.

Chemello, G., Renna, M., Caimi, C., Guerreiro, I., Oliva-Teles, A., Enes, P., Biasato, I., Schiavone, A., Gai, F., Gasco, L., 2020. Partially defatted tenebrio molitor larva meal in diets for grow-out rainbow trout, *Oncorhynchus mykiss* (Walbaum): effects on growth performance, diet digestibility and metabolic responses. Animals 10, 229. https://doi.org/10.3390/ani10020229.

Chemello, G., Biasato, I., Gai, F., Capucchio, M.T., Colombino, E., Schiavone, A., Gasco, L., Pauciullo, A., 2021. Effects of *Tenebrio molitor* larvae meal inclusion in rainbow trout feed: myogenesis-related gene expression and histomorphological features. Ital. J. Anim. Sci. 20, 1211–1221. https://doi.org/10.1080/1828051X.2021.1945959.

Cody, R.M., 1989. Distribution of chitinase and chitobiase in bacillus. Curr. Microbiol. 19, 201–205. https://doi.org/10.1007/BF01570162.

Desai, A.R., Links, M.G., Collins, S.A., Mansfield, G.S., Drew, M.D., Van Kessel, A.G., Hill, J.E., 2012. Effects of plant-based diets on the distal gut microbiome of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 350–353, 134–142. https://doi.org/10.1016/j.aquaculture.2012.04.005.

Dixon, P., 2003. VEGAN, a package of R functions for community ecology. J. Veg. Sci. 14, 927–930. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x.

Dumas, A., Raggi, T., Barkhouse, J., Lewis, E., Weltzien, E., 2018. The oil fraction and partially defatted meal of black soldier fly larvae (*Hermetia illucens*) affect differently growth performance, feed efficiency, nutrient deposition, blood glucose and lipid digestibility of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 492, 24–34. https://doi.org/10.1016/j.aquaculture.2018.03.038.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E., Relman, D.A., 2005. Diversity of the human intestinal microbial flora. Science 308, 1635–1638. https://doi.org/10.1126/science.1110591.

Elia, A.C., Capucchio, M.T., Caldaroni, B., Magara, G., Dörr, A.J.M., Biasato, I., Biasibetti, E., Righetti, M., Pastorino, P., Prearo, M., Gai, F., Schiavone, A., Gasco, L., 2018. Influence of *Hermetia illucens* meal dietary inclusion on the histological traits, gut mucin composition and the oxidative stress biomarkers in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 496, 50–57. https://doi.org/10.1016/j.aquaculture.2018.07.009.

Fu, P.P., Xiong, F., Feng, W.W., Zou, H., Wu, S.G., Li, M., Wang, G.T., Li, W.X., 2019. Effect of intestinal tapeworms on the gut microbiota of the common carp, *Cyprinus carpio*. Parasit. Vectors 12, 252. https://doi.org/10.1186/s13071-019-3510-z.

Gürkök, S., Görmez, A., 2016. Isolation and characterization of novel chitinolytic bacteria. AIP Conf. Proc. 1726, 020017 https://doi.org/10.1063/1.4945843.

Huang, Q., Sham, R.C., Deng, Y., Mao, Y., Wang, C., Zhang, T., Leung, K.M.Y., 2020. Diversity of gut microbiomes in marine fishes is shaped by host-related factors. Mol. Ecol. 29, 5019–5034. https://doi.org/10.1111/mec.15699.

Huyben, D., Vidaković, A., Werner Hallgren, S., Langeland, M., 2019. High-throughput sequencing of gut microbiota in rainbow trout (*Oncorhynchus mykiss*) fed larval and pre-pupae stages of black soldier fly (*Hermetia illucens*). Aquaculture 500, 485–491. https://doi.org/10.1016/j.aquaculture.2018.10.034.

Huyben, D., Rimoldi, S., Ceccotti, C., Montero, D., Betancor, M., Iannini, F., Terova, G., 2020. Effect of dietary oil from *Camelina sativa* on the growth performance, fillet fatty acid profile and gut microbiome of gilthead Sea bream (*Sparus aurata*). PeerJ 8, e10430. https://doi.org/10.7717/peerj.10430.

Ingerslev, H.-C., von Gersdorff Jørgensen, L., Strube, M.L., Larsen, N., Dalsgaard, I., Boye, M., Madsen, L., 2014. The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type. Aquaculture 424–425, 24–34. https://doi.org/10.1016/j.aquaculture.2013.12.032.

IPIFF, 2021. An overview of the European market of insects as feed. 〈https://ipiff.org/publications-position-papers/〉 (Accessed 19 February 2022).

Jiang, M., Xu, M., Ying, C., Yin, D., Dai, P., Yang, Y., Ye, K., Liu, K., 2019. The intestinal microbiota of lake anchovy varies according to sex, body size, and local habitat in Taihu Lake, China. Microbiologyopen 9, e00955. https://doi.org/10.1002/mbo3.955.

Józefiak, A., Nogales-Mérida, S., Rawski, M., Kierończyk, B., Mazurkiewicz, J., 2019. Effects of insect diets on the gastrointestinal tract health and growth performance of Siberian sturgeon (*Acipenser baerii* Brandt, 1869). BMC Vet. Res. 15, 348. https://doi.org/10.1186/s12917-019-2070-y.

Khojasteh, S.M.B., Sheikhzadeh, F., Mohammadnejad, D., Azami, A., 2009. Histological, histochemical and ultrastructural study of the intestine of rainbow trout (*Oncorhynchus mykiss*). World Appl. Sci. J. 6, 1525–1531.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, e1 https://doi.org/10.1093/nar/gks808.

Korlević, M., Šupraha, L., Ljubešić, Z., Henderiks, J., Ciglenečki, I., Dautović, J., Orlić, S., 2016. Bacterial diversity across a highly stratified ecosystem: a salt-wedge Mediterranean estuary. Syst. Appl. Microbiol. 39, 398–408. https://doi.org/10.1016/j.syapm.2016.06.006.

Kreling, V., Falcone, F.H., Kehrenberg, C., Hensel, A., 2020. *Campylobacter* sp.: pathogenicity factors and prevention methods-new molecular targets for innovative antivirulence drugs? Appl. Microbiol. Biotechnol. 104, 10409–10436. https://doi.org/10.1007/s00253-020-10974-5.

Kühlwein, H., Emery, M.J., Rawling, M.D., Harper, G.M., Merrifield, D.L., Davies, S.J., 2013. Effects of a dietary β-(1,3)(1,6)-D-glucan supplementation on intestinal microbial communities and intestinal ultrastructure of mirror carp (*Cyprinus carpio* L.). J. Appl. Microbiol. 115, 1091–1106. https://doi.org/10.1111/jam.12313.

Li, Z., Tran, N.T., Ji, P., Sun, Z., Wen, X., Li, S., 2019. Effects of prebiotic mixtures on growth performance, intestinal microbiota and immune response in juvenile chu's croaker, Nibea coibor. Fish. Shellfish. Immunol. 89, 564–573. https://doi.org/10.1016/j.fsi.2019.04.025.

Marono, S., Loponte, R., Lombardi, P., Vassalotti, G., Pero, M.E., Russo, F., Gasco, L., Parisi, G., Piccolo, G., Nizza, S., Di Meo, C., Attia, Y.A., Bovera, F., 2017. Productive performance and blood profiles of laying hens fed *Hermetia illucens* larvae meal as total replacement of soybean meal from 24 to 45 weeks of age. Poult. Sci. 96, 1783–1790. https://doi.org/10.3382/ps/pew461.

Ojha, S., Bußler, S., Schlüter, O.K., 2020. Food waste valorisation and circular economy concepts in insect production and processing. Waste Manag. 118, 600–609. https://doi.org/10.1016/j.wasman.2020.09.010.

Patterson, M.F., McKay, A.M., Connolly, M., Linton, M., 2010. Effect of high pressure on the microbiological quality of cooked chicken during storage at normal and abuse refrigeration temperatures. Food Microbiol. 27, 266–273. https://doi.org/10.1016/j.fm.2009.10.007.

Pelusio, N.F., Rossi, B., Parma, L., Volpe, E., Ciulli, S., Piva, A., D'Amico, F., Scicchitano, D., Candela, M., Gatta, P.P., Bonaldo, A., Grilli, E., 2020. Effects of increasing dietary level of organic acids and nature-identical compounds on growth, intestinal cytokine gene expression and gut microbiota of rainbow trout (*Oncorhynchus mykiss*) reared at normal and high temperature. Fish. Shellfish. Immunol. 107, 324–335. https://doi.org/10.1016/j.fsi.2020.10.021.

Péron, G., Mittaine, J.-F., Le Gallic, B., 2010. Where do fishmeal and fish oil products come from? An analysis of the conversion ratios in the global fishmeal industry. Mar. Policy 34, 815–820.

Quéméneur, M., Bel Hassen, M., Armougom, F., Khammeri, Y., Lajnef, R., Bellaaj-Zouari, A., 2020. Prokaryotic diversity and distribution along physical and nutrient gradients in the Tunisian coastal waters (South Mediterranean Sea). Front. Microbiol. 11, 593540 https://doi.org/10.3389/fmicb.2020.593540.

Rawski, M., Mazurkiewicz, J., Kierończyk, B., Józefiak, D., 2020. Black soldier fly full-fat larvae meal as an alternative to fish meal and fish oil in Siberian Sturgeon nutrition: the effects on physical properties of the feed, animal growth performance, and feed acceptance and utilization. Animals 10, 2119. https://doi.org/10.3390/ani10112119.

Renna, M., Gasmi-Boubaker, A., Lussiana, C., Battaglini, L.M., Belfayez, K., Fortina, R., 2014. Fatty acid composition of the seed oils of selected *Vicia L.* taxa from Tunisia. Ital. J. Anim. Sci. 13, 308–316. https://doi.org/10.4081/ijas.2014.3193.

Renna, M., Schiavone, A., Gai, F., Dabbou, S., Lussiana, C., Malfatto, V., Prearo, M., Capucchio, M.T., Biasato, I., Biasibetti, E., De Marco, M., Brugiapaglia, A., Zoccarato, I., Gasco, L., 2017. Evaluation of the suitability of a partially defatted black soldier fly (*Hermetia illucens* L.) larvae meal as ingredient for rainbow trout (*Oncorhynchus mykiss* Walbaum) diets. J. Anim. Sci. Biotechnol. 8, 57. https://doi.org/10.1186/s40104-017-0191-3.

Rimoldi, S., Terova, G., Ascione, C., Giannico, R., Brambilla, F., 2018. Next generation sequencing for gut microbiome characterization in rainbow trout (*Oncorhynchus mykiss*) fed animal by-product meals as an alternative to fishmeal protein sources. PLoS One 13, e0193652. https://doi.org/10.1371/journal.pone.0193652.

Rimoldi, S., Gini, E., Iannini, F., Gasco, L., Terova, G., 2019. The effects of dietary insect meal from *Hermetia illucens* prepupae on autochthonous gut microbiota of rainbow trout (*Oncorhynchus mykiss*). Animals 9, 143. https://doi.org/10.3390/ani9040143.

Rimoldi, S., Antonini, M., Gasco, L., Moroni, F., Terova, G., 2021. Intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) may be improved by feeding a *Hermetia illucens* meal/low-fishmeal diet. Fish. Physiol. Biochem. 47, 365–380. https://doi.org/10.1007/s10695-020-00918-1.

Ringø, E., Gatesoupe, F.-J., 1998. Lactic acid bacteria in fish: a review. Aquaculture 160, 177–203. https://doi.org/10.1016/S0044-8486(97)00299-8.

Salas-Leiva, J., Mazón-Suástegui, J.M., Teles, A., Tovar-Ramírez, D., 2020. Structure and predictive metabolic contribution of intestinal microbiota of Longfin yellowtail (*Seriola rivoliana*) juveniles in aquaculture systems. Mol. Biol. Rep. 47, 9627–9636. https://doi.org/10.1007/s11033-020-05970-x.

Sealey, W.M., Gaylord, T.G., Barrows, F.T., Tomberlin, J.K., McGuire, M.A., Ross, C., St-Hilaire, S., 2011. Sensory analysis of rainbow trout, *Oncorhynchus mykiss*, fed enriched black soldier fly prepupae, *Hermetia illucens*. J. World Aquac. Soc. 42, 34–45. https://doi.org/10.1111/j.1749-7345.2010.00441.x.

Skrivanova, E., Marounek, M., Benda, V., Brezina, P., 2006. Susceptibility of *Escherichia coli*, *Salmonella* sp., and *Clostridium perfringens* to organic acids and monolaurin. Vet. Med. 51, 81–88.

St-Hilaire, S., Sheppard, C., Tomberlin, J.K., Irving, S., Newton, L., McGuire, M.A., Mosley, E.E., Hady, R., Sealey, W., 2007. Fly prepupae as a feedstuff for rainbow trout, *Oncorhynchus mykiss*. J. World Aquac. Soc. 38, 59–67. https://doi.org/10.1111/j.1749-7345.2006.00073.x.

Tarnecki, A.M., Burgos, F.A., Ray, C.L., Arias, C.R., 2017. Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. J. Appl. Microbiol. 123, 2–17. https://doi.org/10.1111/jam.13415.

Terova, G., Rimoldi, S., Ascione, C., Gini, E., Ceccotti, C., Gasco, L., 2019. Rainbow trout (*Oncorhynchus mykiss*) gut microbiota is modulated by insect meal from *Hermetia illucens* prepupae in the diet. Rev. Fish. Biol. Fish. 29, 465–486. https://doi.org/10.1007/s11160-019-09558-y.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy. Sci. 74, 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2.

Verdile, N., Pasquariello, R., Scolari, M., Sciré, G., Brevini, T.A.L., Gandolfi, F., 2020. A detailed study of rainbow trout (*Oncorhynchus mykiss*) intestine revealed that digestive and absorptive functions are not linearly distributed along its length. Animals 10, 745. https://doi.org/10.3390/ani10040745.

Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P.B., Welch, T., Wiens, G.D., Snekvik, K., Rawls, J.F., Good, C., 2013. Aquacultured rainbow trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. Appl. Environ. Microbiol. 79, 4974–4984. https://doi.org/10.1128/AEM.00924-13.

Yu, J., Wang, Y., Xiao, Y., Li, X., Xu, X., Zhao, H., Wu, L., Li, J., 2021. Effects of chronic nitrate exposure on the intestinal morphology, immune status, barrier function, and microbiota of juvenile turbot (*Scophthalmus maximus*). Ecotoxicol. Environ. Saf. 207, 111287 https://doi.org/10.1016/j.ecoenv.2020.111287.

Yu, M., Li, Z., Chen, W., Rong, T., Wang, G., Ma, X.J., 2019. *Hermetia illucens* larvae as a potential dietary protein source altered the microbiota and modulated mucosal immune status in the colon of finishing pigs. J. Anim. Sci. Biotechnol. 10, 50. https://doi.org/10.1186/s40104-019-0358-1.

Zeng, A., Tan, K., Gong, P., Lei, P., Guo, Z., Wang, S., Gao, S., Zhou, Y., Shu, Y., Zhou, X., Miao, D., Zeng, F., Liu, H., 2020. Correlation of microbiota in the gut of fish species and water. 3 Biotech 10, 472. https://doi.org/10.1007/s13205-020-02461-5.

Zhang, Z., Fan, X., Peijnenburg, W.J.G.M., Zhang, M., Sun, L., Zhai, Y., Yu, Q., Wu, J., Lu, T., Qian, H., 2021. Alteration of dominant cyanobacteria in different bloom periods caused by abiotic factors and species interactions. J. Environ. Sci. 99, 1–9. https://doi.org/10.1016/j.jes.2020.06.001.

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Black soldier fly and yellow mealworm live larvae for broiler chickens: Effects on bird performance and health status

Sara Bellezza Oddon[1] | Ilaria Biasato[1] | Arianna Imarisio[2] | Miha Pipan[3] | Dominik Dekleva[3] | Elena Colombino[2] | Maria Teresa Capucchio[2] | Marco Meneguz[1] | Stefania Bergagna[4] | Raffaella Barbero[4] | Marta Gariglio[2] | Sihem Dabbou[5,6] | Edoardo Fiorilla[2] | Laura Gasco[1] | Achille Schiavone[2]

[1]Department of Agricultural, Forest and Food Sciences, University of Turin, Grugliasco, Italy
[2]Department of Veterinary Sciences, University of Turin, Grugliasco, Italy
[3]Entomics Biosystems, Cambridge, UK
[4]Veterinary Medical Research Institute for Piemonte, Liguria and Valle d'Aosta, Turin, Italy
[5]Center Agriculture Food Environment, University of Trento, Trento, Italy
[6]Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige, Italy

Correspondence
Ilaria Biasato, Department of Agricultural, Forest and Food Sciences, University of Turin, Grugliasco, Italy.
Email: ilaria.biasato@unito.it

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Abstract
The commercial broiler chicken strains are the result of successful selection programmes. Most of the problems related to welfare arise from the high growth rate and body weight. The use of environmental enrichments in intensive farming could have a positive effect on birds by increasing animal welfare. The aim of the study was to evaluate the effects of *Hermetia illucens* (HI) and *Tenebrio molitor* (TM) live larvae in the diets of broiler chickens on growth performance, carcass yield and health status. A total of 180 four-day-old male broiler chickens (Ross 308) were randomly allotted to 18 pens. Each pen was assigned to one of the three dietary treatments (6 replicates/treatment, 10 birds/replicate) as follows: (i) control diet (C): commercial feed (two feeding phases: starter [4–11 days] and grower [12–38 days]), (ii) HI: C + 5% of the expected daily feed intake (DFI) HI live larvae (calculated on dry matter [DM]) and (iii) TM: C + 5% of DFI TM live larvae (DM). At 39 days of age, birds were slaughtered. Growth performance parameters were overall not affected by dietary treatments, except for the grower phase feed conversion ratio (FCR) and the overall FCR being better in the TM broilers than the others ($p < 0.01$). No differences were observed for slaughtering performance and haematological and serum parameters, except for the spleen relative weight being higher ($p < 0.01$) in the birds administered with larvae when compared to the C group. Gut morphometric indexes and histopathological alterations were not influenced by insect larvae administration. In conclusion, the administration in limited quantities of HI and TM live larvae as environmental enrichment has no negative effects on broiler chicken growth performance and health status. A behavioural study could confirm that live insect larvae represent a novel natural environmental enrichment in broiler farming.

KEYWORDS
black soldier fly, environmental enrichment, growth performance, gut health, insect larvae, poultry, yellow mealworm

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1 | INTRODUCTION

In Europe, poultry industry has progressively moved on towards an industrialized and vertically integrated broiler production, with substantial increase in *per capita* poultry consumption and significant decline of market prices for broilers (Magdelaine et al., 2019). Currently, the commercial broiler chicken strains are the result of successful selection programmes for two important productive characteristics: the rapid growth and the body conformation (Scheuermann et al., 2003). The maximization of the productive efficiency has progressively led to the adoption of intensive indoor systems to carry out the broiler chicken production (Robins & Phillips, 2011). However, the intensive rearing may frequently cause the parallel degradation of the welfare status of the birds, thus being furtherly exacerbated by barren environment (Riber et al., 2018). Therefore, environmental enrichments have recently been proposed as effective tools to create a more diverse rearing habitat for the animals. The environmental enrichment is generally defined as an improvement in the biological functioning of captive animals resulting from modification of their environment (Newberry, 1995). Since in nature birds eat insects in larval, pupal and adult forms, they can be used as environmental and nutritional enrichments in broiler chicken farming (Zuidhof et al., 2003). The recovery of a natural behaviour could therefore have positive effects on the animal welfare and, consequently, on the breeding and final product images that the consumer creates. Consumers are increasingly interested in the issue of animal welfare, as they seem to prefer food products which are perceived to be more 'animal friendly' (Harper & Makatouni, 2002).

The introduction of a natural element, such as the live insect larvae, into a fully artificial breeding could expand the range of behavioural expression and increase the motility of the animals. For this reason, the use of live larvae can be considered a novel, promising environmental enrichment. Indeed, the addition of variable food items (such as the insects) may be attractive for birds and could potentially increase the amount of time spent for the foraging behaviour and, consequently, make animals more active (Koene, 1999; Bizeray et al., 2002).

Currently, the use of insects in animal feed is a topic of growing interest (Gasco et al., 2019). So far, many researchers have focused their attention on the introduction of insect meal in poultry diets. In particular, the available studies investigated the effects of *Tenebrio molitor* (TM) and *Hermetia illucens* (HI) larva meal on broiler chicken growth performance and animal health (Bovera et al., 2016; Biasato et al., 2017; Dabbou et al., 2018; Biasato et al., 2019). In the recent years, the interest has also been directed to the use of live insect larvae as feed ingredients or enrichments in poultry nutrition. For this reason, there are few scientific papers on this issue; trials have been carried out on turkeys (Veldkamp & van Niekerk, 2019) and laying hens (Star et al., 2020) fed HI live larvae, with improved growth performance (in terms of better feed efficiency) having overall been observed. In broiler chickens, on the other hand, the effects of HI live larvae on animal activity and leg health were recently evaluated (Ipema et al., 2020). The authors observed that the activity was numerically higher and lasted for a longer time in broiler chickens fed 10% of larvae and 4 times per day when compared to the control birds (Ipema et al., 2020). According to EU legislation, in particular the Commission Regulation No. 999/2001, the use of live insects in poultry feeding is allowed as the feed ban regarding the use of insect-derived proteins does not apply to live insects (IPIFF, 2020).

Nowadays, the standard evaluation of the bird performance has progressively been accompanied by the concomitant assessment of the gut health dynamics, as a healthy, well-functioning intestine is considered a synonymous of animal health and it represents the main determinant of the animal growth (Kogut & Arsenault, 2016). In particular, the morphometric evaluation of selected gut segments and/or mucosal elements (such as the crypts and the villi)—together with the blood profile and organ histopathological analyses—has previously been resulted to be an effective, easy-to-use approach to simultaneously characterize the gut health and overall health status of broilers (Biasato et al., 2018). However, such analyses have not been performed yet on birds administered with live insect larvae as environmental enrichments.

Based on the above-mentioned background, the goal of this study is to evaluate the effects of the administration of TM and HI live larvae on performance, blood profile, gut morphology and organ histopathology in broiler chickens.

2 | MATERIALS AND METHODS

2.1 | Birds and husbandry

The present trial was performed in the poultry facility of the University of Turin (Italy). The experimental protocol (ID: 814715) was approved by the Bioethical Committee of the University of Turin (Italy). A total of 180 four-day-old male broiler chickens (Ross 308; average initial live weight: 88.6 ± 9.8 g) were randomly allotted to 18 pens. Each pen was 1.20 m wide × 2.20 m long and was covered with rice hulls as litter. The poultry house was equipped with a waterproof floor and walls, completely covered by tiles and provided with an automatic ventilation system. The lighting schedule was 16-h light:8-h darkness for the whole trial. Environmental conditions in the house, temperature and relative humidity, were set according to the Ross guidelines (Aviagen, 2019). The animals and the environmental parameters were daily checked during the whole experimental period.

2.2 | Diets

According to birds' requirements (Aviagen 2019), the basal diet was a commercial feed having two feeding phases: starter (1–11 days; metabolizable energy [ME]: 12.5 MJ/kg and crude protein [CP]: 224 g/kg) and grower (12–38 days; ME: 13.0 MJ/kg and CP: 220 g/kg) (Fa.ma.ar.co SPA). Each pen was assigned to the dietary treatments (6 replicates/treatment, 10 birds/replicate) as follows: (i) control (C), (ii), HI: C + 5% of the expected daily feed intake [DFI] HI live larvae and

TABLE 1 Proximate composition of HI and TM larvae

Chemical composition[a] (as fed basis, %)	HI early instar larvae	HI late instar larvae	TM early instar larvae	TM late instar larvae
DM	25.32	25.32	27.54	27.54
CP	12.01	8.07	16.78	10.82
Ash	3.05	2.00	1.69	0.90
EE	0.42	1.93	0.59	5.50
GE (MJ/kg)	5.03	6.76	5.90	7.65

Abbreviations: CP, crude protein; DM, dry matter; EE, ether extract; GE, gross energy.
[a]Values are reported as mean of duplicate analyses.

(iii) TM: C + 5% of the expected of DFI TM live larvae. The amount of the live larvae was calculated on the dry matter (DM) of the expected DFI reported in the Ross guidelines (Aviagen, 2019). To prevent the chicks from not being able to feed due to the too large size of the larvae, different larvae dimensions were provided in the two growth periods and precisely: early or late instar (length: 0.80 cm ± 0.05 and 1.5 ± 0.05 cm, respectively) for starter and grower period respectively. The commercial diet and the water were distributed ad libitum in all the treatments. For each pen, the daily larvae quantity was placed in two plates and distributed at 11.00 a.m. To avoid the bias, two plates with a known amount of control feed inside were also provided to the C animals to create the same interaction with the operators in all the treatments. When the larvae intake was ended in all the boxes, the leftover C feed was returned in the feeders. Every day, the time spent by birds eating the larvae was recorded in the larvae treatments only by stopwatch, starting from the moment the plates with the larvae were located in the box until the plates were empty. Samples of larvae were periodically collected, killed by freezing (−20°C) and stored to carry out the chemical analyses.

2.3 | Larvae preservation

The quantity of HI and TM larvae needed to satisfy the 5% of the DFI was weekly sent by Entomics Biosystems (Cambridge, UK) and by Italian Cricket Farm (Turin, Italy) respectively.

Since the HI larvae underwent one day of transport, they were shipped along with the substrate to secure feed. Upon arrival, the larvae were separated from the substrate. According to Holmes et al. (2016), the HI larvae were kept in a climatic chamber at 16°C to trigger the diapause mechanism. The diapause is a dynamic process consisting of several successive phase, and it is generally defined as an endogenously arrest of the direct development that proceeds with an alternative programme of physiological events (Koštál, 2006). As far as TM larvae are concerned, the study of Qin & Walker (2005) was taken as reference. In particular, as these authors reported that at 5.5°C, the activity of thermal hysteresis is still found in the haemolymph of TM, a pre-trial (data not reported) to induce diapause was held placing TM larvae at different refrigeration temperatures (6°C, 8°C and 10°C). Once returned at room temperature in all cases, the diapause ended. For this trial, it was then decided to store larvae in a climatic chamber at 6°C.

As viable larva was needed for the chickens to be attracted to the environmental enrichment, prior to administration to birds, the larvae underwent a revitalization phase at a temperature of 28°C for 10 min. In this period of time, they reactivated their metabolism and, consequently, their motility.

2.4 | Chemical analysis

At each arrival, a sample of larvae was stored (−20°C) for subsequent analytical evaluation. The larvae samples were subsequently freeze-dried and ground using cutting mill (MLI 204; Bühler AG). Samples were analysed for: DM (method number 943.01), ash (method number 924.05), crude protein (CP, method number 984.13) and ether extract (EE, method number 2003.05) according to International AOAC (DM, ash, CP: AOAC, 2000; EE; AOAC, 2003). The gross energy (GE) content was determined using an adiabatic calorimetric bomb (C7000; IKA). The HI and TM larvae proximate composition is shown in Table 1.

2.5 | Growth performance

At the beginning of the experimental trial, birds were individually labelled with a wing mark. The live weight (LW) of the animals was recorded at an individual level (4, 11 and 38 days of age) using electronic scales (KERN PLE-N v. 2.2; KERN & Sohn GmbH; d: 0.1). The average daily gain (ADG) and the daily feed intake (DFI) were calculated on pen basis at the end of each growth period (4–11 days; 12–38 days), while the feed conversion ratio (FCR) was calculated on pen basis at the end of each growth period and for the overall experimental trial (4–11 days; 12–38 days; 4–38 days). The FCR was calculated by including in the formula the amount (g) of larvae (based on DM: 25.3% for HI larvae and 27.5% for TM larvae) intake by the birds.

2.6 | Slaughtering procedure and recording

At 39 days of age, after 12 h of feed withdrawal, final LW was recorded. On the basis of the average final LW, 3 birds/pen (18 broilers/diet) were electrically stunned and slaughtered at a commercial

TABLE 2 Effects of the dietary treatments on the growth performance of the broiler chickens (n = 6)

Items	Age (days)	Dietary treatments[a] C	HI	TM	SEM	p Value[b]
LW, g	4	87	87	88	0.38	0.796
	11	220	216	225	3.60	0.603
	38	2488	2527	2452	22.28	0.619
ADG, g/d	4–11	19	18	20	0.49	0.610
	12–38	76	72	80	1.98	0.348
DFI, g/d	4–11	24	22	22	0.67	0.679
	12–38	110	108	103	3.45	0.753
FCR, g/G	4–11	1.25	1.23	1.16	0.02	0.223
	12–38	1.36^{ab}	1.39^{a}	1.32^{b}	0.01	**
	4–38	1.37^{a}	1.38^{a}	1.31^{b}	0.01	**

Abbreviations: ADG, average daily gain; DFI, daily feed intake; FCR, feed conversion ratio; LW, live weight; SEM, standard error of the mean.
[a]Three dietary treatments: C = control; HI: C + 5% of DFI HI larvae; and TM: C + 5% of DFI TM larvae.
[b]**p value < 0.01.

abattoir. The plucked and eviscerated carcasses were obtained, and the head, neck, feet and abdominal fat were removed to obtain the chilled carcass. Then, the weight of heart, spleen, bursa of Fabricius, liver, gut, gizzard, glandular stomach, visceral fat, thigh and breast were immediately recorded and the data were expressed as percentage of LW. The caeca from the slaughtered animals were also isolated and photographed orthogonally with a metric scale (mm). The images were subsequently analysed with the software Image®-Pro Plus software (6.0 version, Media Cybernetics) in order to record the caecal length.

2.7 | Haematological and serum parameters

At slaughter, blood samples were collected from the slaughtered birds. A total of 2.5 ml was placed in an EDTA tube and 2.5 ml in a serum-separating tube. From a drop of blood without anticoagulant, a blood smear was prepared and the May–Grünwald and Giemsa were used as stain (Campbell, 1995). The blood samples previously treated with a 1:200 Natt–Herrick solution were employed for the total red and white blood cell counts through an improved Neubauer haemocytometer. On every slide, one hundred leucocytes, including granular (heterophils, eosinophils and basophils) and non-granular (lymphocytes and monocytes) leucocytes, were counted, and the heterophils-to-lymphocytes (H/L) ratio was calculated (Salamano et al., 2010).

The serum was obtained by keeping the tubes without anticoagulant in a standing position at room temperature for approximately two hours. Later, the serum was separated by centrifugation (700 g for 15 min) and frozen at −80°C until analysis. The total proteins were quantified by means of the 'Biuret method' (Bio Group Medical System kit; Bio Group Medical System), while the electrophoretic pattern of the serum was obtained using a semi-automated agarose gel electrophoresis system (Sebia Hydrasys®). The albumin, alaninoaminotransferase (ALT), aspartate aminotransferase (AST), triglycerides, cholesterol, uric acid and HDL serum concentrations were measured by means of enzymatic methods in a clinical chemistry analyser (Screen Master Touch, Hospitex diagnostics Srl.), while the LDL serum concentration was calculated according to the Friedewald formula as follows: total cholesterol−HDL−(triglycerides/5) (Friedewald et al., 1972).

2.8 | Histomorphological investigations

The slaughtered birds were also submitted to anatomo-pathological investigations. Samples of intestine (duodenum, jejunum and ileum), liver, spleen, thymus and bursa of Fabricius were collected and processed according to Biasato et al. (2017). The processed samples were submitted to morphometric (gut segments) and histopathological (organs) investigations, following the procedures previously described (Biasato et al., 2017). In addition, gut histopathological findings were separately assessed for mucosa (inflammatory infiltrates) and submucosa (inflammatory infiltrates and gut-associated lymphoid tissue [GALT] activation) for each segment. The total score of each gut segment was obtained by adding up the mucosa and submucosa scores, while the total score of each bird was represented by the mean value of the duodenum, jejunum and ileum scores.

2.9 | Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics V26.0.0 software (IBM). Each pen was considered as the experimental unit for the growth performance and larvae intake times (n = 6 per treatment), while the individual bird was used as the experimental unit to analyse the slaughtering performance, the blood parameters, the gut histomorphology and the histopathological features (n = 18 per treatment).

Shapiro–Wilk's test established normality or non-normality of distribution. Growth performance and blood parameters were analysed by means of one-way ANOVA (post hoc test: Bonferroni's multiple comparison test). Both the larvae intake times and the gut histomorphological findings were analysed by fitting a general linear mixed model (GLMM). In the first case, the GLMM allowed the larvae intake minutes to depend on three fixed factors (diet, time and the interaction between the diet and the time). The replicate was included as a random effect to account for repeated measurements on the same pen. In the second case, the GLMM allowed the morphometric indices (Vh, Cd and Vh/Cd, separately) to depend on three fixed factors (diet, intestinal segment and the interaction between the diet and the intestinal segment). Animal was included as a random effect to account for repeated measurements on the same

bird. The interactions between the levels of the fixed factors were evaluated by means of pairwise comparisons. The histopathological scores were analysed by means of the Kruskal–Wallis test (post hoc test: Dunn's multiple comparison test).

The results were expressed as the mean (growth and slaughtering performance, blood parameters and histopathological features) or least square mean (larvae intake times and gut histomorphological findings) and standard error of the mean (SEM). p values ≤0.05 were considered statistically significant.

3 | RESULTS

3.1 | Growth performance

The data relating to the growth performance parameters are shown in Table 2. The final LW (39 days of age), the ADG and the DFI taking into account the larvae intake did not differ among the groups. The FCR in the starter period was also not affected by the dietary treatment. Differently, the grower period FCR significantly differed among the experimental groups, with the TM birds displaying a better feed efficiency than the HI ($p < 0.01$). Significant differences were also identified in the overall FCR calculation, as the TM broilers showed a better FCR than the other dietary treatments ($p < 0.01$).

3.2 | Larva ingestion time

The broilers fed TM larvae showed lower intake times of larvae than the HI birds ($p < 0.05$; Figure 1), with the fastest larva consumption being also recorded during the second week of the trial for both the groups ($p < 0.05$).

TABLE 3 Effects of the dietary treatments on the slaughtering performance of the broiler chickens ($n = 18$)

	Dietary treatments[a]				
Items	C	HI	TM	SEM	p Value[b]
LW (g)	2477	2517	2442	20.13	0.741
Hot carcass yield (%LW)	76.48	76.01	76.07	0.153	0.402
CCW (g)	1853	1841	1879	12.15	0.429
Cold carcass yield (%LW)	74.42	73.94	73.99	0.159	0.402
Breast yield (%CCW)	32.42	32.08	31.83	0.227	0.574
Thigh yield (%CCW)	29.76	29.47	29.02	0.168	0.205
Spleen (%LW)	0.78[a]	0.94[b]	0.88[b]	0.00	**
Liver (%LW)	1.61	1.68	1.67	0.03	0.602
Bursa of Fabricius (%LW)	0.18	0.20	0.19	0.01	0.562
Heart (%LW)	0.42	0.45	0.45	0.01	0.078
Intestine (%LW)	3.25	3.48	3.34	0.05	0.111
Glandular stomach (%LW)	0.30	0.31	0.30	0.00	0.474
Gizzard (%LW)	1.19	1.23	1.28	0.08	0.894
Abdominal fat (%LW)	1.33	1.24	1.29	0.03	0.598
Caecal length (cm)	16.92	17.25	17.14	0.03	0.771

Abbreviations: CCW, cold carcass weight; LW, live weight; SEM, standard error of the mean.

[a]Three dietary treatments: C = control; HI: C + 5% of DFI HI larvae; and TM: C + 5% of DFI TM larvae.

[b]**p value < 0.01.

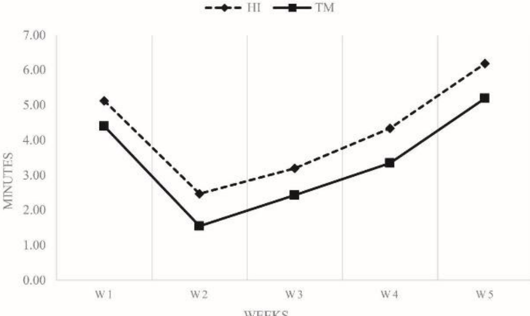

FIGURE 1 Time spent by the broiler chickens on eating the insect larvae

TABLE 4 Effects of dietary treatments on the haematochemical parameters of the broiler chickens ($n = 18$)

Items	Dietary treatments[a]				
	C	HI	TM	SEM	p Value
Erythrocytes (10^6 cell/µl)	2.85	3.13	3.43	1.23	0.165
Leucocytes (10^6 cell/µl)	12.49	14.15	13.13	11.26	0.836
H/L	1.09	1.13	0.91	0.07	0.361
Albumin (g/dl)	1.41	1.46	1.57	0.05	0.396
ALT (U/L)	3.50	2.89	6.00	0.60	0.076
AST (U/L)	372.67	360.33	345.06	15.70	0.779
Cholesterol (mg/dl)	165.22	165.56	162.50	2.43	0.859
Total protein (g/dl)	8.24	8.53	9.36	0.31	0.325
Triglycerides (mg/dl)	39.94	45.61	48.44	1.75	0.131
Uric acid (mg/dl)	3.86	3.47	3.13	0.24	0.468
HDL (mg/dl)	115.50	114.67	111.67	1.45	0.534
LDL (mg/dl)	41.73	41.7	41.14	1.34	0.978

Abbreviations: ALT, alaninoaminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoproteins; H/L, heterophils/lymphocytes; LDL, low-density lipoproteins; SEM, standard error of the mean.

[a]Three dietary treatments: C = control; HI: C + 5% of DFI HI larvae; and TM: C + 5% of DFI TM larvae.

TABLE 5 Intestinal morphometric indices in the broiler chickens in relation to diet and intestinal segment ($n = 18$)

Index	Diet (D)			Intestinal segment (IS)			SEM	p Value[†]		
	C	HI	TM	Duodenum	Jejunum	Ileum		D	IS	D × IS
Vh, mm	1.20	1.27	1.20	1.69^a	1.17^b	0.82^c	0.03	0.129	***	0.866
Cd, mm	0.09	0.10	0.09	0.11^a	0.09^b	0.08^c	0.00	0.057	***	0.576
Vh/Cd	14.01	13.55	12.80	16.61^a	15.21^a	9.46^c	0.47	0.159	***	0.949

Note: The means with different superscript letters (a, b, c) within the same row per fixed effect (i.e. diet, intestinal segment) differ significantly ($p < 0.05$).

Abbreviations: C control, HI C + 5% of DFI HI larvae, TM C + 5% of DFI TM larvae, SEM standard error of the mean, Vh villus height, Cd crypt depth and Vh/Cd villus height-to-crypt depth ratio.

†***p value < 0.01.

3.3 | Slaughtering performance

No significant differences were overall observed for the slaughtering performance among the experimental treatments, except for the spleen relative weight being higher ($p < 0.01$) in the birds administered with insect larvae when compared to the C group (Table 3).

3.4 | Haematological and serum parameters

As reported in Table 4, the haematological and the serum parameters were not influenced by the dietary treatments.

3.5 | Histomorphological investigations

As summarized in Table 5, there was no significant influence of diet and interaction between diet and intestinal segment on the gut morphometric indices. On the contrary, the intestinal segment significantly affected the gut histomorphology ($p < 0.001$). In particular, the duodenum showed greater Vh, Cd and Vh/Cd than the other gut segments ($p < 0.001$), with morphometric indices being also greater in the jejunum when compared to the ileum ($p < 0.001$).

Absent or mild to moderate histopathological alterations were observed in all the organs from all the experimental treatments, as shown in Table 6. Independently of the insect larvae administration, the spleen showed mild, multifocal white pulp hyperplasia, while mild to moderate, multifocal to diffuse follicular depletion was detected in the bursa of Fabricius. Furthermore, the liver showed mild to moderate, multifocal to diffuse vacuolar degeneration of the hepatocytes, as well as mild to moderate, multifocal lymphoplasmacytic infiltrates, whereas in gut mild to moderate, multifocal to diffuse, mucosal or submucosal lymphoplasmacytic infiltrates with or without GALT activation were observed. No alterations were detected in the thymus. However, the insect larvae administration did not affect the histopathological scores.

TABLE 6 Histopathological alterations in the broiler chickens (n = 18)

Items	Dietary treatments[a] C	HI	TM	SEM	p Value
Spleen	0.05	0.32	0.38	0.06	0.062
Thymus	Absence of alterations				
Bursa of Fabricius	0.25	0.17	0.41	0.06	0.308
Liver	1.47	1.55	1.13	0.10	0.197
Gut	1.88	1.22	1.94	0.14	0.069

Note: The data are expressed as the mean of the scores (0 = absence of alterations; 1 = mild alterations; 2 = moderate alterations; 3 = severe alterations). The gut scores resulted from the mean of the three gut segment scores (in turn obtained from the adding of the mucosa and submucosa scores).

Abbreviation: SEM, standard error of the mean.

[a]Three dietary treatments: C = control; HI: C + 5% of DFI HI larvae; and TM: C + 5% of DFI TM larvae.

4 | DISCUSSION

4.1 | Growth performance

During the experimental trial, no mortality was recorded. Since in nature birds eat insects, especially in the first weeks of their life (Bruns, 1960), they can be used as environmental and nutritional enrichments in order to improve animal welfare. Chickens that have access to outdoor zone pick up insects at all life stages and eat them on a voluntary basis, thus meaning that they are evolutionarily adapted to insects as a natural part of their diet (Bovera et al., 2016). This is the reason why the birds of the trial were very eager to eat TM and HI larvae. The consumption of the larvae did not affect the final LW, the ADG, the DFI and the starter period FCR. Despite the absence of a statistical significance, in both the feeding phases the DFI displayed by the C birds was found to be numerically greater than that of the larvae-administered group. Therefore, it is conceivable that the nutrient supply derived from dietary insect larvae supplementation may have partially influenced this growth parameter. In the grower phase, the HI birds showed a similar FCR to the C group, but a worse feed efficiency when compared to the TM broiler chickens. Furthermore, the birds administered with TM larvae showed a better overall FCR when compared to the other groups. The result derived from the comparison of the C group in respect of the HI animals contrasts with those reported in literature. Indeed, in either the turkey or the laying hens trials, in which the quantity of supplied HI larvae was equal to 10% of the DFI, an increase in the feed efficiency was observed (Veldkamp & van Niekerk, 2019; Star et al., 2020). The turkeys fed HI larvae also showed a higher body weight gain and final body weight than the C treatment (Veldkamp & van Niekerk, 2019). On the contrary, the productive parameters in the laying hens (laying rate, egg weight, egg mass and mortality rate) were not affected by the larvae administration (Star et al., 2020). Moreover, previous studies have uncovered that the consumption of larvae up to 5% of the dietary DM has a neutral effect on the growth rate (Ipema et al., 2020). The reduction of FCR in the TM broilers may result from the proximate composition of the larvae. Indeed, in the late instar, the TM larvae of the present study were characterized by a greater quantity of DM, CP and EE than the HI larvae (27.54, 10.82, 5.50; 25.32, 8.07 and 1.93 respectively). Therefore, due to its composition, the TM larvae appear to be more nutritious than the HI larva and its effect appeared in the second period of growth when, proportionally, the quantity of larvae administered became more conspicuous.

4.2 | Larva ingestion time

The assessment of the larva ingestion time revealed that broiler chickens were very eager to eat live larvae, especially the TM larvae. The TM broilers showed lower intake times of larvae than the HI birds (3.45 and 4.27 min, respectively), with the fastest larva consumption being also recorded during the second week of the trial for both the groups. Since in the first week the animals needed to adapt to the new feed provided, the larva ingestion time was longer than the second week. In the last three weeks, the intake time slowed down in relation to the increase in body mass of the birds and, consequently, the increased difficulty of movement. The daily assessment of the larvae intake times represents an innovative parameter. In the study conducted with turkeys, in which HI larvae were provided as 10% of the DFI, the authors observed that the larvae were consumed within two minutes after provision (Veldkamp & van Niekerk, 2019). This result is in contrast to the broilers' intake times of the first, third and fourth week, but is similar to the second week (2.17 min). Apart from the different bird species, the discrepancies between the results may be due to dissimilar methods of measuring the intake time—as Veldkamp & van Niekerk (2019) did not indicate the exact registration start moment—and the type of larvae administration.

4.3 | Slaughtering performance

The slaughtering performance of the broiler chickens was overall not influenced by the dietary treatments. On the contrary, the spleen weight was found to be significantly higher in the larvae-fed animals than in the C group. Indeed, the study showed a reactivity of the spleen white pulp in all the groups, with numerically heavier spleen being identified in the treated animals in comparison with the control birds. This outcome can be related to the chitin, a polysaccharide existing in insect that exerts an immunostimulant effect (Gasco et al., 2018). Bovera et al. (2016) have reached the same conclusion, since the authors observed spleen weight gain in animals fed TM meal and associated the results with the antimicrobial and antifungal chitin properties. Furthermore, more stressed animals have a lower spleen weight due to the corticosterone effect, which depresses lymphoid organs growth (Bovera et al., 2016). Consequently, the birds fed insect larvae could be in a situation of greater welfare than the C group.

4.4 | Haematological and serum parameters

Prior to this study, no research has focused its attention on evaluating the effects of live larvae on broilers' blood parameters. From a general point of view, blood tests represent useful indicators of the health and metabolic status of the birds. In particular, the assessment of blood biochemical parameters, such as total protein, albumin, AST, ALT, cholesterol, triglycerides, uric acid and others, can serve as diagnostic tool for identifying specific metabolic disturbances occurring in the main organs (Nunes et al., 2018). Considering that the haematological and serological analyses led to similar results among the experimental treatments, it is reasonable to hypothesize that the insect larvae administration has no negative effects on bird metabolic status. Furthermore, the blood values that have been taken into consideration in the present study are within the broiler chickens physiological ranges (Lumej, 2008). The results obtained are also in agreement with those of Biasato et al. (2018) and Dabbou et al. (2018), in which no effect on blood traits was observed in broiler chickens fed with increasing level of insect (HI or TM) meal inclusion (inclusion range 5%–15%).

4.5 | Histomorphological investigations

So far, no histomorphological investigations have been performed in poultry reared with live insect larvae as environmental enrichments. The broiler chickens administered with insects of the present study showed similar gut morphology when compared to the C birds, thus probably revealing no significant effects of the live larvae on the intestinal digestion and absorption properties. Differently, increasing inclusion levels of TM (Biasato et al., 2018) and HI (Dabbou et al., 2018) meals in diets for broiler chickens have previously been reported to deeply worsen the gut morphology of the birds (in terms of shorter villi, deeper crypts and reduced Vh/Cd), also determining a significant impairment in their growth performance (in terms of increased FCR). Since the intestinal morphometric indices resulted herein unaffected, the improvement in the feed efficiency observed in the TM broilers was probably related to the better nutritional profile of the TM larvae in comparison with the HI species.

The insect larvae administration in the broiler chickens of the current trial did not lead to the development of significant histopathological alterations, thus confirming the safety of including insects and insect-based products in poultry nutrition. Indeed, dietary insect meal inclusion has already been reported to not exert a negative influence on the histological traits of the birds (Biasato et al., 2018; Dabbou et al., 2018).

5 | CONCLUSION

In conclusion, based on the results obtained in the present study, the 5% of the expected DFI administration of HI and TM live larvae has no negative effects on broiler chicken growth performance, with a positive impact on feed efficiency of TM-fed broilers being even observed. Moreover, it has been shown that different insect species in larval stage can differently affect the production parameters of the birds. The live larvae, thanks to its chitin component, could have immunostimulant properties and, therefore, can positively influence the broiler chicken health. Future behavioural and welfare studies will be required to confirm that live insect larvae can be used as natural environmental enrichment in intensive poultry farming.

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ANIMAL WELFARE STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes and feed legislation.

ORCID

Sara Bellezza Oddon https://orcid.org/0000-0001-8942-0682
Ilaria Biasato https://orcid.org/0000-0002-8855-4248
Arianna Imarisio https://orcid.org/0000-0002-6137-0377
Miha Pipan https://orcid.org/0000-0001-8391-5924
Dominik Dekleva https://orcid.org/0000-0003-1685-541X
Elena Colombino https://orcid.org/0000-0002-6371-2000
Maria Teresa Capucchio https://orcid.org/0000-0002-1068-0551
Marco Meneguz https://orcid.org/0000-0001-8007-2044
Stefania Bergagna https://orcid.org/0000-0001-5390-4108
Raffaella Barbero https://orcid.org/0000-0003-4497-6382
Marta Gariglio https://orcid.org/0000-0001-5224-8604
Sihem Dabbou https://orcid.org/0000-0002-3525-1614
Edoardo Fiorilla https://orcid.org/0000-0002-0173-1118
Laura Gasco https://orcid.org/0000-0002-1829-7936
Achille Schiavone https://orcid.org/0000-0002-8011-6999

REFERENCES

AOAC International. (2000). *Official methods of analysis of AOAC International* (17th ed.). AOAC International.

AOAC International. (2003). *Official methods of analysis of AOAC International* (17th ed., 2nd revision). AOAC International.

Aviagen. (2019). *Ross 308 Broiler nutrition specification*. Retrieved from http://eu.aviagen.com/tech-center/download/1339/Ross308-308FF-BroilerPO2019-EN.pdf

Biasato, I., Gasco, L., De Marco, M., Renna, M., Rotolo, L., Dabbou, S., Capucchio, M. T., Biasibetti, E., Tarantola, M., Bianchi, C., Cavallarin, L., Gai, F., Pozzo, L., Dezzutto, D., Bergagna, S., & Schiavone, A. (2017). Effects of yellow mealworm larvae (*Tenebrio molitor*) inclusion in diets for female broiler chickens: implications for animal

health and gut histology. *Animal Feed Science and Technology*, *234*, 253–263. https://doi.org/10.1016/j.anifeedsci.2017.09.014

Biasato, I., Gasco, L., De Marco, M., Renna, M., Rotolo, L., Dabbou, S., Capucchio, M. T., Biasibetti, E., Tarantola, M., Sterpone, L., Cavallarin, L., Gai, F., Pozzo, L., Bergagna, S., Dezzuto, D., Zoccarato, I., & Schiavone, A. (2018). Yellow mealworm larvae (*Tenebrio molitor*) inclusion in diets for male broiler chickens: Effects on growth performance, gut morphology, and histological findings. *Poulty Science*, *97*, 540–548. https://doi.org/10.3382/ps/pex308

Biasato, I., Ferrocino, I., Grego, E., Dabbou, S., Gai, F., Gasco, L., Cocolin, L., Capucchio, M. T., & Schiavone, A. (2019). Gut microbiota and mucin composition in famale broiler chickens fed diets including yellow mealworm (*Tenebrio molitor*, L.). *Animals*, *9*, 213. https://doi.org/10.3390/ani9050213

Bizeray, D., Estevez, I., Leterrier, C., & Faure, J. M. (2002). Influence of increased environmental complexity on leg condition, performance, and level of fearfulness in broilers. *Poultry Science*, *81*, 767–773. https://doi.org/10.1093/ps/81.6.767

Bruns, H. (1960). The economic importance of birds in forests. *Bird study*, *7*, 193–208. https://doi.org/10.1080/00063656009475972

Bovera, F., Loponte, R., Marono, S., Piccolo, G., Parisi, G., Iaconisi, V., Gasco, L., & Nizza, A. (2016). Use of *Tenebrio molitor* larvae meal as protein source in broiler diet: Effect on growth performance, nutrient digestibility, and carcass and meat traits. *Journal of Animal Science*, *94*, 639–647. https://doi.org/10.2527/jas2015-9201

Campbell, T. W. (1995). *Avian hematology and cytology* (2nd ed.). Iowa State University Press.

Dabbou, S., Gai, F., Biasato, I., Capucchio, M. T., Biasibetti, E., Dezzutto, D., Meneguz, M., Plachà, I., Gasco, L., & Schiavone, A. (2018). Black soldier fly defatted meal as a dietary protein source for broiler chickens: Effects on growth performance, blood traits, gut morphology and histological features. *Journal of Animal Science and Biotechnology*, *9*(49), 1–10.

Friedewald, W. T., Levy, R. I., & Fredrickson, D. S. (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clinical Chemistry*, *18*, 499–502. https://doi.org/10.1093/clinchem/18.6.499

Gasco, L., Finke, M., & van Huis, A. (2018). Can diets containing insects promote animal health? *Journal of Insects as Food and Feed*, *4*, 1–4. https://doi.org/10.3920/JIFF2018.x001

Gasco, L., Biasato, I., Dabbou, S., Schiavone, A., & Gai, F. (2019). Animals fed insect-based diets: State-of-the-art on digestibility, performance and product quality. *Animals*, *9*, 170. https://doi.org/10.3390/ani9040170

Harper, G. C., & Makatouni, A. (2002). Consumer perception of organic food production and farm animal welfare. *British Food Journal*, *104*, 287–299. https://doi.org/10.1108/00070700210425723

Holmes, L. A., VanLaerhoven, S. L., & Tomberlin, J. K. (2016). Lower temperature threshold of black soldier fly (Diptera: Stratiomyidae) development. *Journal of Insects as Food and Feed*, *2*, 255–262. https://doi.org/10.3920/JIFF2016.0008

Ipema, A. F., Gerrits, W. J. J., Bokkers, E. A. M., Kemp, B., & Bolhuis, J. E. (2020). Provisioning of live black soldier fly larvae (*Hermetia illucens*) benefits broiler activity and leg health in a frequency- and dose-dependent manner. *Applied Animal Behaviour Science*, *230*, 105082. https://doi.org/10.1016/j.applanim.2020.105082

IPIFF. (2020). *The insect sector milestones towards sustainable food supply chains*. Retrieved from https://ipiff.org/the-insect-sector-milestones-towards-sustainable-food-supply-chains/

Koene, P. (1999). When feeding is just eating. How do farm and zoo animals use their spare time? In D. van der Heide, E. A. Huisman, E. Kanis, J. W. M. Osse, & M. W. A. Verstegen (Eds.), *Proceedings 5th Zodiac Symposium Wageningen, The Netherlands*, 13-19.

Kogut, M. H., & Arsenault, R. J. (2016). The new paradigm in food animal production. *Frontiers in Veterinary Science*, *3*, 10–13. https://doi.org/10.3389/fvets.2016.00071

Koštál, V. (2006). Eco-physiological phases of insect diapause. *Journal of Insect Physiology*, *52*, 113–127. https://doi.org/10.1016/j.jinsphys.2005.09.008

Lumej, J. T. (2008). Avian clinical biochemistry. In J. J. Kaneko, J. W. Harwey, & M. L. Bruss (Eds.), *Clinical biochemistry of domestic animals*. Elsevier Academic Press.

Magdelaine, P., Spiess, M. P., & Valceschini, E. (2019). Poultry meat consumption trends in Europe. *Wolrd's Poultry Science Journal*, *64*, 56–64. https://doi.org/10.1017/S0043933907001717

Newberry, R. C. (1995). Environmental enrichment: Increasing the biological relevance of captive environments. *Applied Animal Behaviour Science*, *44*, 229–243. https://doi.org/10.1016/0168-1591(95)00616-Z

Nunes, R. V., Broch, J., Wachholz, L., de Suoza, C., Damasceno, J. L., Oxford, J. H., Bloxham, D. J., Billard, L., & Pesti, G. M. (2018). Choosing sample sizes for various blood parameters of broiler chickens with normal and non-normal observations. *Poultry Science*, *97*, 3746–3754. https://doi.org/10.3382/ps/pey217

Qin, W., & Walker, V. K. (2005). *Tenebrio molitor* antifreeze protein gene identification and regulation. *Gene*, *367*, 142–149. https://doi.org/10.1016/j.gene.2005.10.003

Riber, A. B., Van De Weerd, H. A., De Jong, I. C., & Steenfeldt, S. (2018). Review of environmental enrichment for broiler chickens. *Poultry Science*, *97*, 378–396. https://doi.org/10.3382/ps/pex344

Robins, A., & Phillips, C. J. C. (2011). International approaches to the welfare of meat chickens. *World's Poultry Science Journal*, *67*, 251–269. https://doi.org/10.1017/S0043933911000341

Salamano, G., Mellia, E., Tarantola, M., Gennero, M. S., Doglione, L., & Schiavone, A. (2010). Acute phase proteins and heterophil:lymphocyte ratio in laying hens in different housing systems. *Veterinary Record*, *167*, 749–751. https://doi.org/10.1136/vr.c5349

Scheuermann, G. N., Bilgili, S. F., Hess, J. B., & Mulvaney, D. R. (2003). Breast muscle development in commercial broiler chickens. *Poultry Science*, *82*, 1648–1658. https://doi.org/10.1093/ps/82.10.1648

Star, L., Arsiwalla, T., Molist, F., Leushuis, R., Dalim, M., & Paul, A. (2020). Gradual provision of live black soldier fly (*Hermetia illucens*) larvae to older laying hens: Effect on production performance, egg quality, feather condition and behavior. *Animals*, *10*(2), 216. https://doi.org/10.3390/ani10020216

Veldkamp, T., & van Niekerk, T. G. C. M. (2019). Live black soldier fly larvae (*Hermetia illucens*) for turkey poults. *Journal of Insects as Food and Feed*, *5*, 301–311. https://doi.org/10.3920/JIFF2018.0031

Zuidhof, M. J., Molnar, C. L., Morley, F. M., Wray, T. L., Robinson, F. E., Khan, B. A., Al-Ani, L., & Goonewardene, L. A. (2003). Nutritive value of house fly (*Musca domestica*) larvae as a feed supplement for turkey poults. *Animal Feed Science and Technology*, *105*, 225–230. https://doi.org/10.1016/S0377-8401(03)00004-X.

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EDITED BY
Vincent M. Cassone,
University of Kentucky, United States

REVIEWED BY
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University of Lincoln, United Kingdom
Sabine G. Gebhardt-Henrich,
University of Bern, Switzerland
Xavier Averós,
Neiker Tecnalia, Spain

*CORRESPONDENCE
Ilaria Biasato,
ilaria.biasato@unito.it

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Welfare implications for broiler chickens reared in an insect larvae-enriched environment: Focus on bird behaviour, plumage status, leg health, and excreta corticosterone

Ilaria Biasato[1]*, Sara Bellezza Oddon[1], Giulia Chemello[2], Marta Gariglio[3], Edoardo Fiorilla[3], Sihem Dabbou[4], Miha Pipan[5], Dominik Dekleva[5], Elisabetta Macchi[3], Laura Gasco[1] and Achille Schiavone[3]

[1]Department of Agricultural, Forest and Food Sciences, University of Turin, Turin, Italy, [2]Department of Life and Environmental Sciences, Marche Polytechnic University, Ancona, Italy, [3]Department of Veterinary Sciences, University of Turin, Turin, Italy, [4]Center Agriculture Food Environment, University of Trento, Trento, Italy, [5]Entomics Biosystems, Cambridge, United Kingdom

The use of insect live larvae as environmental enrichment has recently been proposed in broiler chickens, but the concomitant administration of black soldier fly (BSF) and yellow mealworm (YM) has never been tested yet. Therefore, the present study aims to evaluate the effects of live BSF and YM larvae as environmental enrichments for broiler chickens by means of plumage status, behaviour, leg health, and excreta corticosterone metabolites (CM). A total of 180 4-day old male Ross 308 broiler chickens were randomly distributed in 3 experimental treatments (6 replicates/treatment, 10 birds/replicate) and fed for 35 days as follows: 1) control (C, commercial feed), 2) BSF: C + 5% of the expected daily feed intake [DFI] live BSF larvae and 3) YM: C + 5% of the expected DFI live YM larvae. Feathering, hock burn (HB) and footpad dermatitis (FPD) scores (end of the trial), as well as behavioural observations (beginning of the trial [T0] and every 11 days [T1, T2 and T3] during morning, larvae intake and afternoon) through video recordings, were assessed, and excreta samples collected to evaluate the CM. Feathering, HB and FPD scores, and excreta CM were unaffected by insect live larvae administration ($p > 0.05$). In the morning, the insect-fed birds displayed higher stretching, wing flapping, ground pecking (at T1 and T3), as well as lower preening (at T1 and T2), than the C group ($p < 0.05$). During the larvae intake, higher scratching, wing flapping and ground pecking, as well as lower stretching, preening and laying down, were observed in the insect-fed (scratching, stretching and laying down) or YM-fed (wing flapping, ground pecking and preening) groups than the C birds ($p < 0.05$). In the afternoon, insect live larvae administration increased wing flapping (YM) and laying down (BSF and YM), as well as decreased ground pecking (YM, $p < 0.05$). In conclusion, the administration of insect live larvae as environmental enrichment (especially YM) was capable of positively influencing the bird

welfare through the stimulation of foraging behaviour, increase in activity levels, and reduction in bird frustration, without affecting the plumage status, leg health, and excreta CM.

KEYWORDS

black soldier fly, broiler chickens, environmental enrichment, welfare, yellow mealworm

Introduction

Insects are nowadays recognized as excellent biofactories for their peculiar ability to valorise a wide spectrum of waste materials by nutrition upcycling, which allows obtaining edible high-quality micro- and macro-nutrients that can be incorporated in the animal feed chain (Gasco et al., 2020). The so-obtained insect larvae are, indeed, predominantly fractionated to obtain meals and oils, which can efficiently be utilized to replace the conventional protein and lipid sources in monogastric diets (Ravi et al., 2020). However, the scientific research recently carried on revealed that insect live larvae may also potentially reach an interesting market share in the form of environmental enrichments for either poultry (Pichova et al., 2016; Veldkamp and van Niekerk, 2019; Ipema et al., 2020; Star et al., 2020; Bellezza Oddon et al., 2021; Tahamtani et al., 2021) or pigs (Ipema et al., 2021a; Ipema et al., 2021b).

Environmental enrichment can be defined as a modification of the rearing environment of captive animals aimed at improving their biological functioning and stimulating their species-specific behaviours (Newberry, 1995). The enrichment strategies currently available for broiler chickens can be grouped in 2 main categories: 1) "point-source objects", which are enrichment objects/devices that are generally limited in size and whose use is often restricted to a single or a few locations in an animal enclosure; and 2) more complex enriched environments designed to meet the key behavioural needs of the animals within them (i.e., outdoor access) (Riber et al., 2018). Among the "point-source objects", the provision of food items to stimulate the bird foraging activity represents one of the most practical and effective enrichment techniques, as search for various types of food resources on the litter has been reported to increase foraging and movement in broiler chickens (Pichova et al., 2016; Ipema et al., 2020). Such increase in overall activity levels may have implications for the intensive farming, where the fast growth rates and the high body weights are the main cause of leg problems and lameness in broilers, thus, in turn, deeply limiting their ability to move (Reiter and Bessei, 2009). Furthermore, as fast-growing broilers spend between 60 and 80% of their time sitting (de Jong and Gunnink, 2018), contact dermatitis (i.e., hock burns, breast burns and foot pad dermatitis) may also frequently occur, as a consequence of continuing contact and pressure of the skin of the breast, hocks and feet against humid and soiled bedding (Ekstrand et al., 1998). The limited space and the absence of environmental stimuli of the commercial conditions can also impair broiler welfare by limiting the possibility to perform intrinsically motivated behaviours and diminishing activity levels, thus, in turn, furtherly increasing the occurrence of leg problems (Vasdal et al., 2019), and the susceptibility to abdominal dermatitis, plumage soiling and feet and hock dermatitis (Bruce et al., 1990; Opengart et al., 2018).

Black soldier fly (BSF) and yellow mealworm (YM) live larvae provision has recently been proposed as promising food environmental enrichment to promote welfare in broiler chickens, with increased activity and foraging behaviour (as a result of the search for larvae on the ground), and reduced occurrence of hock burns and lameness (as a result of the increased activity) being observed in the administered birds (Pichova et al., 2016; Ipema et al., 2020). Welfare assessment in broiler chickens is usually object of a multiperspective approach, as heterogeneous parameters (such as plumage status, hock burns and footpad dermatitis, lameness, behavioural patterns, and excreta corticosterone) are commonly evaluated (Weimer et al., 2018; Giersberg et al., 2021; Iannetti et al., 2021; Lourenço da Silva et al., 2021). Despite beneficial live insect larvae-related effects on bird behaviour and feathering scores having recently been highlighted in either turkeys (Veldkamp and van Niekerk, 2019) or laying hens (Star et al., 2020; Tahamtani et al., 2021), data about modulation of plumage status and excreta corticosterone in broiler chickens reared in live insect larvae-enriched environment are still missing. Furthermore, no studies assessing the effects of the concomitant administration of BSF and YM live larvae as environmental enrichments are currently available in poultry.

Therefore, the present study aims to investigate the effects of BSF and YM live larvae as environmental enrichments for broiler chickens, assessing the implications for bird welfare by means of behaviour, plumage status, leg health, and excreta corticosterone metabolites (CM).

Materials and methods

Birds and experimental design

The experimental design of the present study is reported in details by Bellezza Oddon et al. (2021), as the current research is part of the same project and was performed using the same birds. In order to provide a brief summary, a total of 180 4-day old male Ross

TABLE 1 Description of the broiler ethogram (frequency and duration behaviours) considered in the present study.

Frequency behaviour	Definition
Scratching	Scraping of the litter with the claws (Ipema et al., 2020)
Preening	Grooming of own feathers with beak (Ipema et al., 2020)
Trotting	Increasing walking step with head high and breast out (Veldkamp and van Niekerk, 2019)
Pecking pen mate	Pecking movements directed at the body or beak of a pen mate (Ipema et al., 2020)
Stretching	Stretching one wing together with the leg at the same side or both wings upward and forward (Martin et al., 2005)
Chasing	One hen chasing another, with fast running, no vocalisations, no hopping and no wing flapping (Sokołowicz et al., 2020)
Wing flapping	Number of wing beats, often while the bird is standing on the toes (Martin et al., 2005)
Shaking	Body/wing shake when the plumage is not in order (Martin et al., 2005)
Dust bathing	Sitting and performing: vertical wing-shaking, body shaking, litter pecking and/or scratching, bill raking, side and head rubbing (van Hierden et al., 2002)
Allopreening	Social preening (Kenny et al., 2017)
Duration behaviours	**Definition**
Walking	Taking one or more step (Webster and Hurnik, 1990)
Preening	Grooming of own feathers with beak (Ipema et al., 2020)
Standing still	Standing on the feet with extended legs (Webster and Hurnik, 1990)
Ground pecking	Pecking at the litter with the head in lower position than the rump (van Hierden et al., 2002)
Lying down	Sitting position (Webster and Hurnik, 1990)

308 broiler chickens were randomly allotted to 3 experimental treatments (6 replicate pens/treatment, 10 birds/treatment) as follows: 1) control (C), where a commercial feed only was provided (two feeding phases: starter [4–11 days] and grower-finisher [12–38 days]; ii), BSF, where the C diet was supplemented with 5% of the expected daily feed intake [DFI] of BSF live larvae (calculated on dry matter [DM]); and 3) YM, where the C diet was supplemented with 5% of the expected DFI of YM live larvae (DM). The starter commercial feed was characterized by 12.5 MJ/kg metabolizable energy (ME) and 224 g/kg crude protein (CP), while the grower feed contained 13.0 MJ/kg ME and 220 g/kg CP (Fa.ma.ar.co SPA, Cuneo, Italy). The pens were 1.20 m wide × 2.20 m long (bird density at the end of the growth: 10 kg/m^2). The daily amount of live larvae was distributed to all the pens in two plates at the same hour (11.00 a.m.) and 7 days/week for the whole trial (35 days). To avoid any potential bias, two plates with a known amount of control feed inside were also provided to the C animals to create the same interaction with the operators in all the treatments, and there was also a visual separation among the pens (Bellezza Oddon et al., 2021).

Feathering score

At the end of the experimental trial, all the birds were given feathering scores for back, breast, wing, under-wing and tail using scores of 1–5 for feather coverage as follows: score 1, minimal coverage (<25% coverage); score 2, 25%–50% coverage; score 3, 50%–75% coverage; score 4, >75% coverage; and score 5, complete coverage (Lai et al., 2010).

Behavioural observations

The behavioural observations were carried out using video recordings. A total of 3 pens/treatment were filmed for 5 min in the morning (9.00–9.05 a.m.), 5 min during the larvae intake (11.00–11.05 a.m.) and 5 min in the afternoon (6.00–6.05 p.m.) at the beginning of the trial (T0) and every 11 days until the end of the experiment (T1, T2 and T3). The recorded videos were analysed by the Behavioural Observation Research Interactive Software (BORIS, v 7.9.7) (Friard and Gamba, 2016). The considered behaviours were divided in two categories: the frequency (point event) and the duration (state event) behaviours (Table 1). The frequency behaviours were evaluated as the number of times that a specific behaviour occurred in the pen during the 5 min periods of observations. The duration behaviours were, instead, assessed as the percentage of the 5 min periods of observations that 4 identified subjects in the pen (named as alpha, beta, gamma and delta) spent performing a specific behaviour.

Feet and hock health assessment

The feet and hocks of the broiler chickens were examined at the end of the experimental trial in order to assess the incidence

and the severity of the footpad dermatitis (FPD) and the hock burns (HB). The FPD was scored as follows: 0 = no lesion, slight discoloration of the skin or healed lesion; 1 = mild lesion, superficial discoloration of the skin and hyperkeratosis; and 2 = severe lesion, affected epidermis, blood scabs, haemorrhages and severe swelling of the skin (Ekstrand et al., 1998). Differently, the HB were scored as follows: 0 = no lesion; 1 = superficial, attached (single) lesion or several single superficial or deep lesions ≤0.5 cm; 2 = deep lesion >0.5 cm to ≤1 cm or superficial lesion >0.5 cm; 3 = deep lesion >1.0 cm; 4 = whole hock extensively altered (Louton et al., 2020).

Excreta corticosterone analysis

At the beginning of the trial (T0) and every 11 days (after the video recordings of the administration of the insect live larvae) until the end of the experiment (T1, T2, and T3), all the birds from each pen were housed in wire-mesh cages (100 cm width × 50 cm length) for 120 min to collect fresh excreta samples. After collection, the excreta samples were pooled, immediately frozen at −20°C until corticosterone analysis, and processed according to Palme et al. (2013) and Costa et al. (2016). In particular, the excreta were freeze-dried and ground using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland). A total of 0.25 g of the samples were placed into an extraction tube with 3 ml of ether and stored at −20°C for 1 h. After this time, the aliquots were mixed for 3 min through multivortex and the supernatant was recovered and transferred in a new tube. The tubes were then placed at 50°C for 14 h to obtain a dried extract. Lastly, excreta CM were analysed with a multi species enzyme immunoassay kit (Arbor Assay®, Ann Arbor, MI, United States) developed for serum, plasma, saliva, urine, extracted faecal samples, and tissue culture media. All of the analyses were performed in duplicate. The inter- and intra-assay coefficients of variation were less than 10% (7% and 9%, respectively). The sensitivity of the assay was 11.2 ng/g of excreta. All of the samples were analysed at multiple dilutions (1:4, 1:8, 1:16, and 1:32) and all the regression slopes were parallel to the standard curve ($r^2 = 0.979$).

Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics V28.0.0 software (IBM, Armonk, NY, United States). The pen was considered as the experimental unit for the plumage status, behaviour, and excreta CM analyses, while the bird was used for the assessment of the leg health. Shapiro-Wilk's test established normality or non-normality of distribution of both the data and the residuals. The feathering scores were analysed by fitting a generalized linear mixed model (GLMM) that allowed them to depend on linear predictors (diet, time, and their interaction) through a negative binomial response probability distribution with a nonlinear link function (log). The mean scores of each body area were included in the statistical model. A GLMM was also fit to allow the behaviour data to depend on the same linear predictors through a Poisson loglinear distribution (frequency behaviours) or a gamma probability distribution with a nonlinear (log) link function (duration behaviours). The total number of times that the specific frequency behaviours occurred in the pen, as well as the mean percentage of time that the 4 identified subjects of the pen spent performing the specific duration behaviours, were included in the corresponding statistical models. Frequency behaviours occurring less than 0.5 times on average per period of observation were excluded from the GLMM. The excreta CM were also analysed by fitting a GLMM that allowed them to depend on the same linear predictors through a gamma probability distribution with a nonlinear link function (log). The mean CM resulting from the duplicate analysis was included in the statistical model. The replicate was included as a random effect to account for repeated measurements on the same pen, and the interactions between the levels of the fixed factors were evaluated by means of pairwise contrasts. The HB and FPD scores were analysed by means of Kruskal-Wallis (post-hoc test: Dunn's Multiple Comparisons Test). The results were expressed as least square mean (plumage status, behaviour, and excreta CM) or mean (leg health) and standard error of the mean (SEM). p values ≤0.05 were considered statistically significant.

Results

Feathering score

The feathering scores of the broiler chickens of the current research are summarized in Table 2. The administration of both the BSF and the YM live larvae did not influence the feathering scores of the birds ($p = 0.545$). On the contrary, the feathering scores depended on the body area ($p < 0.001$). In particular, the back showed better scores when compared to the other body areas, with breast, under-wing and tail furtherly displaying greater scores than the wing ($p < 0.001$). No diet × body area interaction was also identified ($p = 0.237$).

Behaviour analysis

Frequency behaviours of the broiler chickens of the present study are summarized in Table 3 and Figures 1–3. In the morning, stretching and wing flapping were influenced by both the insect live larvae administration and the time ($p < 0.001$), but no diet × time interaction was identified ($p = 0.686$ and $p = 0.220$, respectively). In details, the insect-fed broiler chickens performed more stretching and wing flapping than the C group ($p < 0.001$), and, independently of diet, a

TABLE 2 Feathering score of the broiler chickens depending on diet, body area and their interaction.

	Diet (D)			Body area (B)					SEM		*p*-value			Wald test		
	C	BSF	YM	Back	Breast	Wing	Under-wing	Tail	D	B	D	B	D×B	D	B	D×B
Score, n	1.18	1.16	1.21	3.19^a	1.00^b	0.73^c	1.00^b	0.99^b	0.03	0.05	0.545	<0.001	0.237	1.214	854.780	8.010

C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. Means with superscript letters (a, b, c) denote significant differences ($p < 0.05$).

reduction (stretching) and an increase (wing flapping) of such behaviours was overall observed along the experimental trial ($p < 0.001$ and $p = 0.010$, respectively). The wing flapping frequency also abruptly decreased at T3 when compared to the other experimental times ($p = 0.010$). Preening depended on time only, with an increase being overall identified along the experimental trial, but an abrupt reduction at T3 ($p = 0.001$). On the contrary, no influence of insect live larvae administration or diet × time interaction were highlighted ($p = 0.102$ and $p = 0.110$, respectively). Allopreening, pecking pen mate and shaking behaviours did not depend on any of the considered variables (diet: $p = 0.549$, $p = 1.000$ and $p = 0.001$, respectively; time: $p = 0.549$, $p = 0.290$ and $p = 0.100$, respectively; diet × time: $p = 0.404$, $p = 1.000$ and $p = 1.000$, respectively). During the larvae intake, scratching and wing flapping behaviours were influenced by insect live larvae administration only ($p = 0.025$ and $p < 0.001$, respectively). In particular, the insect-fed broilers performed more scratching in comparison with the C birds ($p = 0.025$), while increased frequency in wing flapping was identified in the YM group only ($p < 0.001$). Differently, no influence of time ($p = 0.070$ or $p = 0.661$, respectively) or diet × time interaction ($p = 0.662$ and $p = 0.508$, respectively) were identified. Preening and stretching behaviours were influenced by either the insect live larvae administration or the time ($p < 0.001$). In particular, the insect-fed birds displayed less preening and stretching than the C broilers, with the YM group furtherly showing reduced stretching when compared to the BSF-fed birds ($p < 0.001$). Furthermore, independently of diet, preening and stretching frequencies progressively increased in the last 11 days of the experimental trial ($p < 0.001$). On the contrary, no diet × time interaction was highlighted ($p = 0.057$ and $p = 0.104$, respectively). Trotting and shaking behaviours depended on time only, with trotting frequency progressively decreasing in the last 11 days of the experimental trial ($p < 0.001$), and shaking displaying the opposite trend ($p < 0.001$). Differently, no influence of insect live larvae administration ($p = 0.098$ or $p = 0.687$, respectively) or diet × time interaction ($p = 1.000$ and $p = 0.492$, respectively) were identified. Allopreening and pecking pen mate behaviours did not depend on any of the considered variables (diet: $p = 0.624$ and $p = 0.105$, respectively; time: $p = 1.000$ and $p = 0.624$, respectively; diet × time: $p = 1.000$ and $p = 1.000$, respectively). In the afternoon, a diet × time interaction was observed for wing flapping only ($p < 0.001$). In details, the YM-fed broiler chickens performed more wing flapping than the other groups at T2 and T3 only ($p < 0.001$), while the C birds displayed higher wing flapping than the HI group at T1 ($p < 0.05$, Figure 3). On the contrary, preening, stretching and shaking behaviours depended on time only, with increasing frequencies being highlighted along the experimental trial ($p < 0.001$). On the contrary, no influence of insect live larvae administration ($p = 0.770$, $p = 0.302$ or $p = 0.378$, respectively) or diet × time interaction ($p = 0.127$, $p = 0.106$ and $p = 0.052$, respectively) were highlighted. Allopreening was not influenced by any of the considered variables (diet: $p = 1.000$; time: $p = 0.527$; diet × time: $p = 0.527$).

Duration behaviours of the broiler chickens of the current research are summarized in Table 4 and Figure 4–6. In the morning, a diet × time interaction was observed for both the ground pecking and the preening ($p < 0.001$ and $p = 0.006$, respectively). In particular, higher ground pecking was observed in the insect-fed broilers than the C group at T1 and T3 only ($p < 0.001$, Figure 4), whereas the C birds spent more time preening in comparison with the other groups or BSF group alone at T1 and T2, respectively ($p = 0.006$, Figure 4). Walking depended on either the insect live larvae administration or the time ($p = 0.001$ and $p < 0.001$, respectively). In details, the BSF birds spent more time walking when compared to the C group ($p < 0.001$), and, independently of diet, less walking was progressively observed along the experimental trial ($p < 0.001$). Differently, no diet × time interaction was identified ($p = 0.186$). Standing still and laying down behaviours were influenced by time only ($p < 0.001$ and $p = 0.045$, respectively). In particular, broiler chickens spent less time standing still along the experimental trial ($p < 0.001$), with an increase in laying down being also observed ($p < 0.05$). During the larvae intake, ground pecking and laying down depended on insect live larvae administration only ($p < 0.001$). In particular, the YM-fed birds displayed higher and lower, respectively, ground pecking and preening than the other groups, with either the BSF or the YM broilers spending less time laying down when compared to the C group ($p < 0.001$). On the contrary, no influence of time ($p = 0.703$ and $p = 0.190$, respectively) or diet × time interaction ($p = 0.118$ and $p = 0.141$, respectively) were highlighted. Preening was influenced by both the insect live larvae administration and the time ($p < 0.001$ and $p = 0.001$, respectively). In details, the YM-fed birds displayed lower preening than the other groups ($p < 0.001$), and,

TABLE 3 Frequency behaviours of the broiler chickens depending on diet, time and their interaction.

	Diet (D)			Time (T)				SEM		p-value			Wald test		
	C	BSF	YM	T0	T1	T2	T3	D	T	D	T	D×T	D	T	D×T
Morning															
Scratching, n	<0.5 times of occurrence														
Preening, n	9.72	8.35	9.96	5.45^{a}	8.05^{b}	26.70^{c}	6.44^{a}	9.34	0.88	0.102	0.001	0.110	4.980	13.913	4.342
Allopreening, n	0.00	0.00	0.00	0.00	0.00	0.00	0.48	0.00	0.03	0.549	0.549	0.404	1.200	1.200	1.810
Trotting, n	<0.5 times of occurrence														
Stretching, n	2.07^{a}	4.08^{b}	4.74^{b}	2.92^{a}	2.89^{ab}	3.31^{ab}	4.91^{b}	0.26	0.71	<0.001	<0.001	0.686	45.794	18.871	0.842
Pecking pen mate, n	0.00	0.00	0.00	0.00	0.00	0.67	0.00	0.00	0.17	1.000	0.290	1.000	0.000	2.412	0.000
Chasing, n	<0.5 times of occurrence														
Dust bathing, n	<0.5 times of occurrence														
Wing flapping, n	0.00^{a}	1.67^{b}	2.77^{c}	1.88^{b}	1.44^{b}	6.38^{a}	0.00^{c}	0.21	0.25	<0.001	0.010	0.220	136.671	9.294	3.030
Shaking, n	0.00	0.00	0.00	0.00	0.00	0.87	0.00	0.00	0.03	1.000	0.100	1.000	0.000	4.280	0.000
During larvae intake															
Scratching, n	0.33^{a}	2.28^{b}	2.52^{b}	1.20	1.21	1.06	1.49	0.27	0.41	0.025	0.070	0.662	7.416	9.787	0.825
Preening, n	13.05^{a}	3.59^{b}	4.74^{b}	4.00^{a}	3.85^{a}	7.32^{b}	7.89^{b}	1.16	0.98	<0.001	<0.001	0.057	75.693	206.003	5.716
Allopreening, n	0.00	0.00	0.00	0.40	0.42	0.00	0.00	0.00	0.07	0.624	1.000	1.000	0.240	0.000	0.000
Trotting, n	0.00	0.00	0.00	1.31^{a}	1.46^{a}	0.00^{b}	0.00^{b}	0.00	0.13	0.098	<0.001	1.000	4.645	39.095	0.000
Stretching, n	4.89^{a}	2.00^{b}	1.39^{c}	1.70^{a}	1.88^{a}	2.65^{b}	2.71^{b}	0.52	0.29	<0.001	<0.001	0.104	16.280	15.192	4.532
Pecking pen mate, n	0.00	0.00	0.53	0.00	0.00	0.00	0.00	0.07	0.00	0.105	0.624	1.000	4.950	0.786	0.000
Chasing, n	<0.5 times of occurrence														
Dust bathing, n	<0.5 times of occurrence														
Wing flapping, n	3.15^{a}	2.63^{a}	4.73^{b}	3.45	3.61	3.86	2.81	0.31	0.79	<0.001	0.661	0.508	82.131	0.829	1.356
Shaking, n	0.00	0.00	1.01	0.00^{a}	0.00^{a}	0.00^{a}	2.27^{b}	0.22	0.03	0.687	<0.001	0.492	0.752	84.592	0.472
Afternoon															
Scratching, n	<0.5 times of occurrence														
Preening, n	7.39	8.12	8.80	4.61^{a}	6.96^{b}	8.77^{c}	15.17^{d}	1.15	0.90	0.770	<0.001	0.127	0.522	143571.734	4.125
Allopreening, n	0.00	0.00	0.00	0.00	0.53	0.00	0.53	0.00	0.17	1.000	0.527	0.527			
Trotting, n	<0.5 times of occurrence														
Stretching, n	3.73	5.46	4.26	1.59^{a}	4.61^{b}	6.31^{b}	8.33^{c}	0.66	0.52	0.302	<0.001	0.106	1.891	49.443	5.231

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TABLE 3 (*Continued*) Frequency behaviours of the broiler chickens depending on diet, time and their interaction.

	Diet (D)			Time (T)				SEM		*p*-value			Wald test		
	C	BSF	YM	T0	T1	T2	T3	D	T	D	T	D×T	D	T	D×T
Pecking pen mate, n	<0.5 times of occurrence														
Chasing, n	<0.5 times of occurrence														
Dust bathing, n	<0.5 times of occurrence														
Wing flapping, n	0.00	0.00	1.25	1.30	1.52	0.00	1.19	0.09	0.31	0.309	0.888	0.001	2.346	0.237	14.554
Shaking, n	0.00	0.00	0.00	0.00^{a}	0.00^{a}	0.76^{b}	1.37^{c}	0.00	0.09	0.378	<0.001	0.052	1.947	20.694	5.975

C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33. Means with superscript letters (a, b, c, d) denote significant differences ($p < 0.05$).

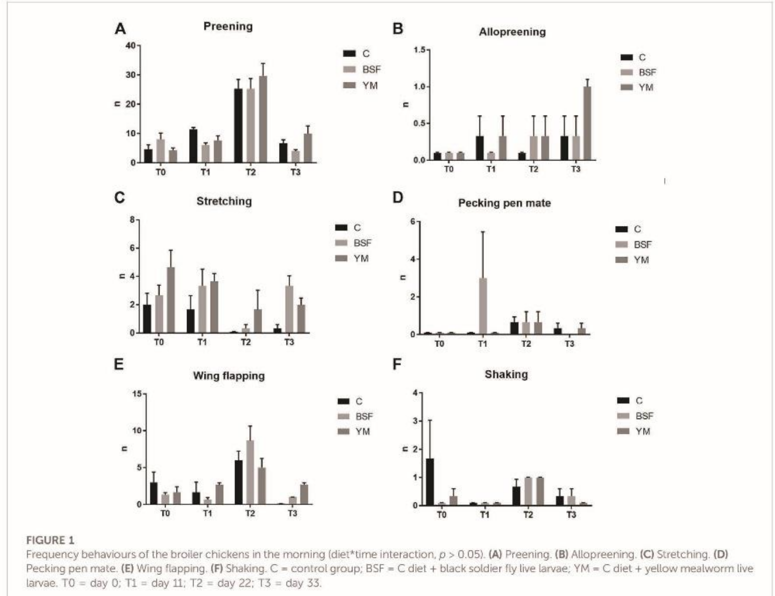

FIGURE 1
Frequency behaviours of the broiler chickens in the morning (diet*time interaction, $p > 0.05$). **(A)** Preening. **(B)** Allopreening. **(C)** Stretching. **(D)** Pecking pen mate. **(E)** Wing flapping. **(F)** Shaking. C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

independently of diet, preening duration was reduced in the last 11 days of the experimental trial ($p = 0.001$). Differently, no diet×time interaction was identified ($p = 0.060$). On the contrary, no influence of insect live larvae administration or diet × time interaction were observed ($p = 0.208$ and $p = 0.077$, respectively). Standing still did not depend on any of the considered variables

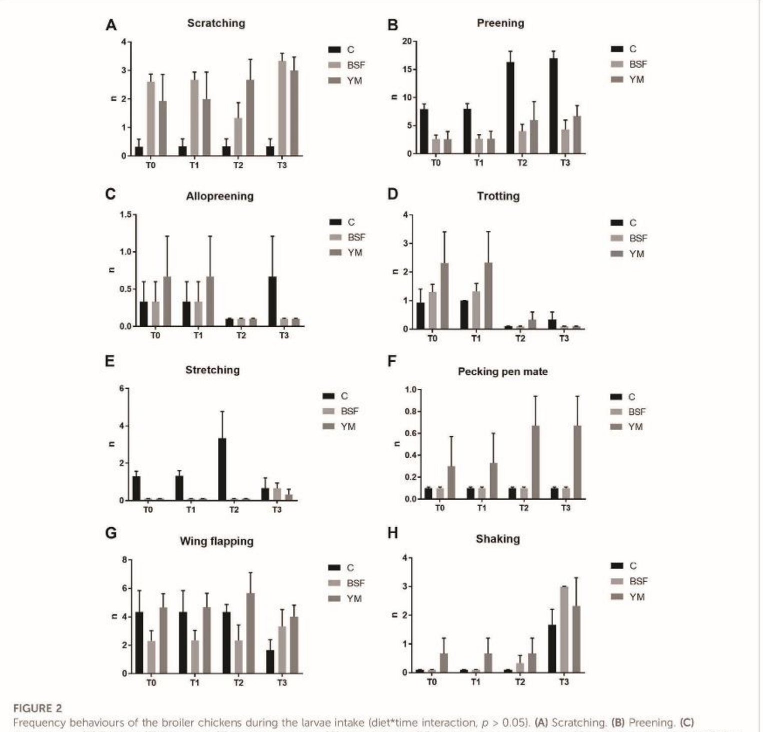

FIGURE 2
Frequency behaviours of the broiler chickens during the larvae intake (diet*time interaction, $p > 0.05$). **(A)** Scratching. **(B)** Preening. **(C)** Allopreening. **(D)** Trotting. **(E)** Stretching. **(F)** Pecking pen mate. **(G)** Wing flapping. **(H)** Shaking. C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

(diet: $p = 0.218$; time: $p = 0.710$; diet × time: $p = 0.058$). In the afternoon, the insect-fed birds showed higher laying down in comparison with the C group at T3 only (diet × time interaction, $p < 0.001$; Figure 6). Ground pecking behaviour depended on insect live larvae administration, with the YM-fed broiler chickens spending less time ground pecking than the other groups ($p < 0.001$). On the contrary, no influence of time or diet × time interaction were highlighted ($p = 0.110$ and $p = 0.571$, respectively). Finally, walking, standing still and preening behaviours were influenced by time only ($p < 0.001$), with broiler chickens spending less time walking and standing still, as well as more time preening, along the experimental trial ($p < 0.001$). Differently, no influence of insect live larvae administration ($p = 0.678$, $p = 0.414$ and $p = 0.285$, respectively) or diet × time interaction ($p = 0.112$, $p = 0.215$ and $p = 0.116$, respectively) were observed.

Feet and hock health assessment

The administration of BSF and YM live larvae did not influence either the HB (H = 3.644; C: 0.37 ± 0.09; BSF: 0.73 ± 0.15; YM: 0.77 ± 0.17) or the FPD (H = 2.603; C: 0.60 ± 0.15; BSF: 0.60 ± 0.14; YM: 0.33 ± 0.11) scores ($p = 0.162$ and $p = 0.272$, respectively).

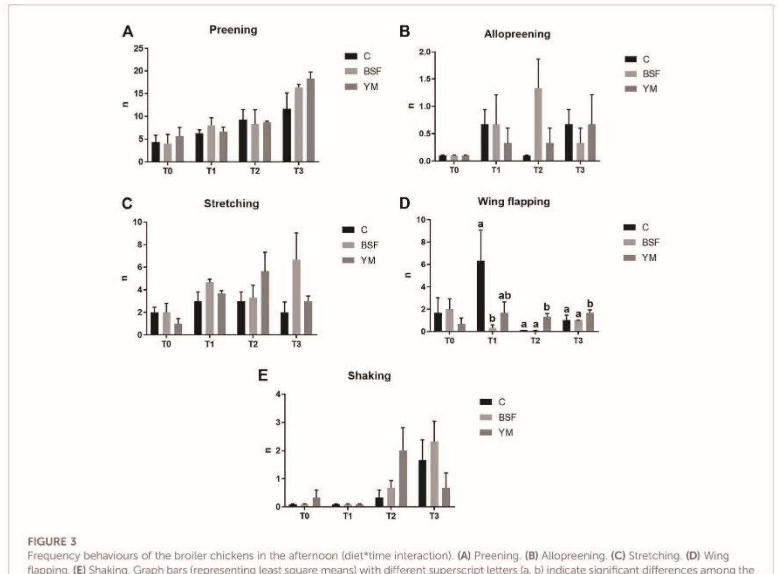

FIGURE 3
Frequency behaviours of the broiler chickens in the afternoon (diet*time interaction). (A) Preening. (B) Allopreening. (C) Stretching. (D) Wing flapping. (E) Shaking. Graph bars (representing least square means) with different superscript letters (a, b) indicate significant differences among the experimental treatments within the experimental times. C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

Excreta corticosterone

The excreta CM of the broiler chickens of the present study are summarized in Table 5 and Figure 7. The administration of BSF and YM live larvae did not affect the excreta CM of the broiler chickens of the current research ($p = 0.684$). Similarly, no time-related effects or diet × time interactions were identified ($p = 0.288$ and $p = 0.369$, respectively).

Discussion

Feathering score

The administration of neither the BSF nor the YM live larvae was able to improve the feathering scores of the broiler chickens of the present study. Previous research highlighted a tendency towards improvement or a significant improvement in feather damage of BSF live larvae-fed turkey poults and laying hens, respectively (Veldkamp and van Niekerk, 2019; Star et al., 2020). Such improvement has been related to a reduction in the aggressive pecking directed at the back and tail base, as a consequence of the re-direction of this behaviour towards the floor and away from feathers (Veldkamp and van Niekerk, 2019). However, since the aggressive pecking displayed by the broilers of the current research was not influenced by the administration of either the BSF or the YM live larvae, it is reasonable that feather conditions were unaffected as well. Independently of the utilization of the insect larvae, the back and the wing of the birds showed the best and the worst feather coverage, respectively. Little information is currently available on the feathering scores of the different body parts in broiler chickens (Lai et al., 2010; Mahmoud et al., 2015; Sevim et al., 2022), with the totality of the body areas being not always assessed (Sevim et al., 2022), or the authors reporting a mean body score only (Mahmoud et al., 2015). Lai et al. (2010) previously identified similar feathering scores among the different body regions of broiler chickens, while a clear

TABLE 4 Duration behaviours of the broiler chickens depending on diet, time and their interaction.

	Diet (D)			Time (T)				SEM		p-value			Wald test		
	C	BSF	YM	T0	T1	T2	T3	D	T	D	T	D×T	D	T	D×T
Morning															
Ground pecking, time %	2.59^{a}	7.12^{ab}	6.11^{b}	7.62^{a}	2.64^{c}	5.55^{b}	4.88^{b}	0.89	0.49	<0.001	<0.001	<0.001	101.932	366.984	235.8011
Walking, time %	4.74^{a}	5.99^{b}	3.95^{ab}	14.43^{a}	8.00^{b}	2.76^{b}	1.66^{c}	0.45	0.88	0.001	<0.001	0.186	14.706	128.630	3.362
Standing still, time %	23.52	19.91	19.89	41.98^{a}	27.22^{b}	8.28^{c}	20.71^{b}	2.67	3.21	0.573	<0.001	0.355	1.115	37.646	2.070
Laying down, time %	46.45	51.23	56.03	29.36^{a}	52.59^{ab}	73.21^{b}	60.24^{b}	3.49	6.56	0.055	0.045	0.107	5.793	6.184	16.710
Preening, time %	7.91^{a}	4.72^{b}	7.34^{b}	2.02^{a}	5.24^{b}	12.40^{d}	6.98^{c}	0.84	0.88	0.019	0.004	0.006	7.906	11.024	10.203
During larvae intake															
Ground pecking, time %	1.61^{ab}	1.66^{a}	2.52^{b}	2.10	2.06	2.14	2.52	0.85	0.58	<0.001	0.703	0.118	93.006	0.146	5.674
Walking, time %	3.29	4.24	4.78	5.58^{a}	5.50^{a}	5.64^{a}	2.92^{b}	0.63	0.24	0.208	<0.001	0.077	3.139	38.806	5.132
Standing still, time %	15.32	17.12	20.45	18.58	18.20	17.86	17.15	1.93	1.53	0.218	0.710	0.058	3.050	0.139	6.008
Laying down, time %	75.27^{a}	33.65^{b}	44.08^{b}	43.39	42.78	44.69	51.88	4.55	2.84	<0.001	0.190	0.141	251.827	1.714	3.918
Preening, time %	6.82^{a}	4.33^{a}	2.20^{b}	5.75^{a}	5.90^{a}	6.40^{a}	2.53^{b}	1.01	0.66	<0.001	0.001	0.060	140.920	12.020	5.640
Afternoon															
Ground pecking, time %	8.12^{a}	6.13^{a}	2.87^{b}	6.26	4.51	6.09	4.34	1.00	0.85	<0.001	0.110	0.571	19.931	4.421	1.120
Walking, time %	5.17	5.25	4.42	23.65^{a}	6.18^{b}	2.17^{c}	1.86^{d}	0.65	1.03	0.678	<0.001	0.112	0.778	18619.759	4.980
Standing still, time %	16.85	14.79	15.15	45.74^{a}	16.45^{b}	7.05^{c}	11.08^{b}	1.95	1.64	0.414	<0.001	0.215	1.761	1013.777	3.165
Laying down, time %	36.04^{a}	52.31^{b}	64.22^{b}	17.65^{a}	60.99^{b}	75.61^{b}	73.60^{b}	5.55	5.09	<0.001	<0.001	<0.001	370.193	44.580	486.225
Preening, time %	2.50	2.96	3.97	1.67^{a}	2.67^{b}	2.78^{b}	7.32^{c}	0.42	0.47	0.285	<0.001	0.116	2.510	11294.008	5.125

C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33. Means with superscript letters (a, b, c, d) denote significant differences ($p < 0.05$).

separation between the back and the other body areas was herein outlined. The poor feather coverage of the breast can reasonably be attributed to the clear predominance of laying down behaviour in the whole behavioural time budget of the birds, while wing, under-wing and tail feather damage may be related to the progressively increase in preening frequency and duration along the experimental trial. Indeed, wing and tail—along with breast—represent the plumage areas receiving preferred attention from the birds during preening (Duncan and Wood-Gush, 1972). A significant role of the genetic selection—which aims at growth of meat and not feathers—cannot be excluded as well.

Behaviour analysis

The variations in the behavioural repertoire of the broiler chickens of the present study share several similarities between the morning and the moment of the larvae intake, while the afternoon was characterized by different behavioural patterns. During the morning and the larvae intake, birds receiving the insect live larvae spent more time ground pecking (with a statistical significance being detected at T1 and T3 only, as a consequence of the higher SEM of T2) and performing increased scratching behaviour when compared to the non-supplemented animals. This clear stimulation of a more natural behaviour such as foraging [characterized by ground pecking and/or scratching (Ipema et al., 2020)] has already been observed in turkey poults and broiler chickens administered with BSF live larvae (Veldkamp and van Niekerk, 2019; Ipema et al., 2020). Scattering food items on the litter (such insects) or using different bedding materials (sand, moss-peat, or oat husks) have previously been reported to stimulate foraging behaviour in broiler chickens (Arnould et al., 2004; Baxter and O'Connell, 2016; Pichova et al., 2016). However, similar environmental enrichments (such as whole wheat, wood shavings, rice hulls or straw pellets) are not capable of exerting an analogous effect (Bizeray et al., 2002; Shields et al., 2005; Toghyani et al., 2010; Jordan et al., 2011; Baxter and O'Connell, 2016; Pichova et al., 2016), thus suggesting that birds have a clear preference for certain types of substrates (Riber et al., 2018). Indeed, the motivational significance behind each food-based enrichment represents the main driver of the behavioural changes (Pichova et al., 2016), and the insect larvae—as alive, moving and part of the natural diet of birds—seem to be highly interesting for poultry (Bokkers

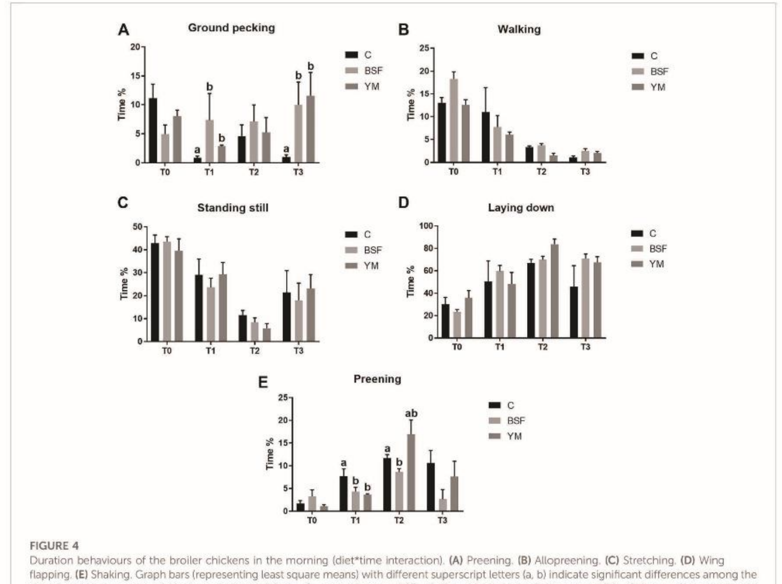

FIGURE 4
Duration behaviours of the broiler chickens in the morning (diet*time interaction). **(A)** Preening. **(B)** Allopreening. **(C)** Stretching. **(D)** Wing flapping. **(E)** Shaking. Graph bars (representing least square means) with different superscript letters (a, b) indicate significant differences among the experimental treatments within the experimental times. C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

and Koene, 2002; Bruce et al., 2003; Ipema et al., 2020). The same motivational significance reasonably determined the increase in the activity levels of the insect-fed broiler chickens of the current research as well, as demonstrated by the increased frequency of stretching and wing flapping behaviours (the latter being mainly detected in the YM-fed birds), the increased time spent for walking and performing wing flapping, and the decreased time spent for laying down. An analogous scenario was also underlined in broilers and laying hens administered with BSF or YM live larvae as environmental enrichment (Pichova et al., 2016; Ipema et al., 2020; Star et al., 2020). It is, however, interesting to notice that the increase in stretching was observed in the morning only, while during the larvae intake such behaviour actually decreased. This may reasonably be related to the parallel increase in scratching and wing flapping behaviours. Another peculiar aspect to highlight is the reduced frequency (independently of time) and duration (mainly with BSF, as a consequence of the higher SEM of the YM group) preening displayed by the insect-fed birds of the present study. Preening, as it keeps plumages well-groomed by distributing lipid-rich oils from uropygial glands and removing parasites (Delius, 1988), could take a large time budget (~13%) out of the total behaviour repertoire of domestic fowl (Dawkins, 1989). However, overall time spent preening and number of preening bouts could give useful information about environment appropriateness for birds (Li et al., 2020). Indeed, absence of environmental stimuli (i.e., cages) stimulates the birds to spend more time preening (Delius, 1988) or to perform short-term and frequent preening (Duncan, 1998), as a sign of boredom and frustration. Therefore, the administration of insect live larvae may reduce such negative feelings in broilers. In the afternoon, birds receiving YM live larvae spent less time ground pecking than the other groups, whereas either the BSF- or the YM-fed broilers showed an increased duration of laying down behaviour (with a statistical significance being detected at T3 only, as a consequence of the higher SEM of T1 and T2). This may indicate that the need for foraging was fully rewarded during the morning and the larvae

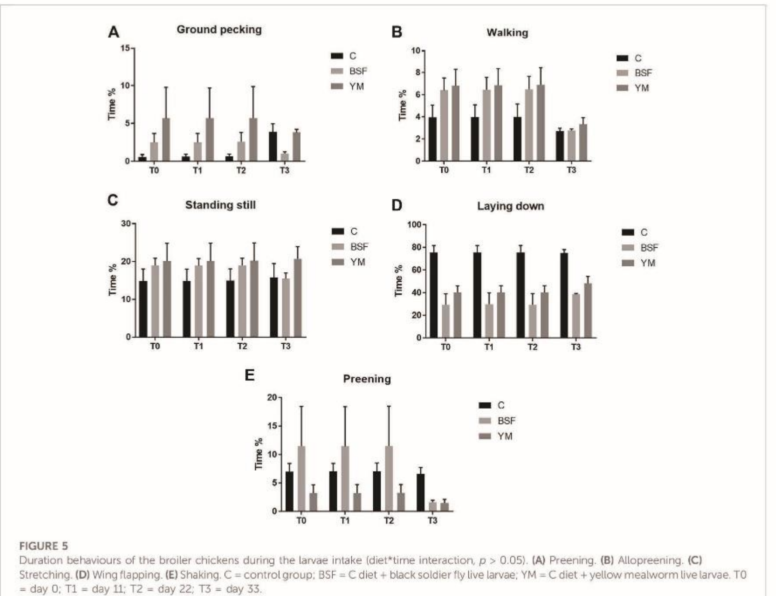

FIGURE 5
Duration behaviours of the broiler chickens during the larvae intake (diet*time interaction, $p > 0.05$). (A) Preening. (B) Allopreening. (C) Stretching. (D) Wing flapping. (E) Shaking. C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

intake, and that the overall increased activity observed in the first part of the day predisposed the birds to rest in the afternoon. However, the wing flapping frequency remained higher in the YM-fed broiler chickens when compared to the other groups (with a statistical significance being detected in the last third of the experimental trial only, as a consequence of the higher SEM of T1).

Independently of the administration of the insect live larvae, the broiler chickens of the present study displayed less active behaviours (i.e., ground pecking, walking and standing still), as well as more passivity (i.e., laying down), with increasing age. This is in agreement with previous research on broilers (Bokkers and Koene, 2003; Castellini et al., 2016; Ipema et al., 2020; Jacobs et al., 2021), where the rapid increase in body weights leads to poor mobility and, in turn, inhibits their ability to express certain behaviours (Bokkers, 2004; Castellini et al., 2016). The overall increase in preening may similarly be attributed to frustration related to poor mobility (Bokkers and Koene, 2003). On the contrary, other active behaviours such as stretching, shaking and wing flapping increased with increasing age of birds. It is, however, important to underline that fast-growing broilers are motivated to perform the normal behavioural repertoire of chickens, even after 6 weeks of age and despite being hampered by the high body weights (Bokkers, 2004). Furthermore, as behaviours are performed in sitting position rather than in standing position with increasing age (Bokkers, 2004), it is reasonable to identify an increase in behaviours that birds can easily perform when laying down.

As a final aspect to consider, the use of YM live larvae yielded slightly more pronounced effects on bird behaviour (especially in terms of stimulation of foraging and increase in activity levels) than the BSF ones. Considering that the broiler chickens of the current research spent less time consuming the YM live larvae when compared to BSF (Bellezza Oddon et al., 2021), it is possible to speculate a bird preference towards the larvae of this insect

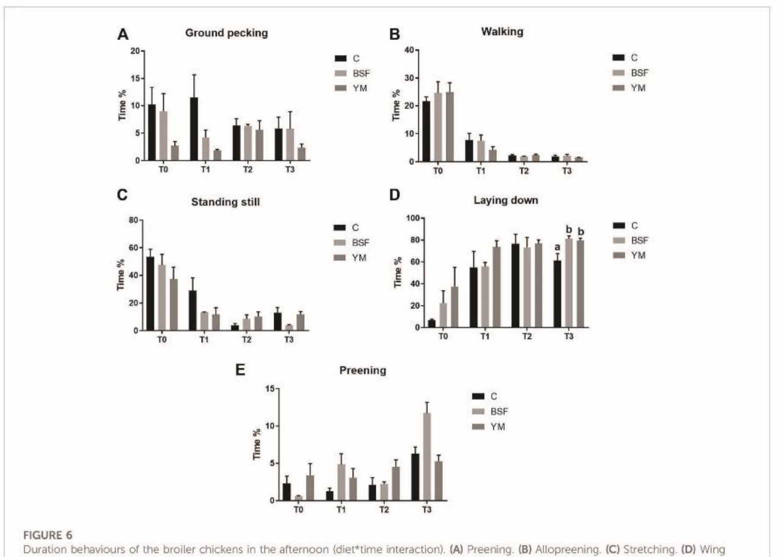

FIGURE 6
Duration behaviours of the broiler chickens in the afternoon (diet*time interaction). **(A)** Preening. **(B)** Allopreening. **(C)** Stretching. **(D)** Wing flapping. **(E)** Shaking. Graph bars (representing least square means) with different superscript letters (a, b) indicate significant differences among the experimental treatments within the experimental times. C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

TABLE 5 Excreta CM of the broiler chickens depending on diet, time and their interaction.

	Diet (D)			Time (T)				SEM		*p*-value			Wald test		
	C	BSF	YM	T0	T1	T2	T3	D	T	D	T	D×T	D	T	D×T
CM, ng/g	2855.8	2955.6	3079.4	3210.3	2978.2	3024.4	2641.4	181.1	209.2	0.684	0.288	0.369	0.382	1.284	1.108

C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

species. However, further studies are needed to confirm this hypothesis.

Feet and hock health assessment

Similarly to what was observed for the feathering scores, the HB and the FPD scores of the broiler chickens of the current research were not influenced by the administration of either the BSF or the YM live larvae. Ipema et al. (2020) highlighted that FPD occurrence was not affected by insect live larvae provision, whereas the larvae-administered birds displayed less HB when compared to the C birds. However, considering that FPD incidence has been reported to be influenced only in the first 3 weeks of age in turkey poults (Veldkamp and van Niekerk, 2019), it is reasonable that a single evaluation may not be enough

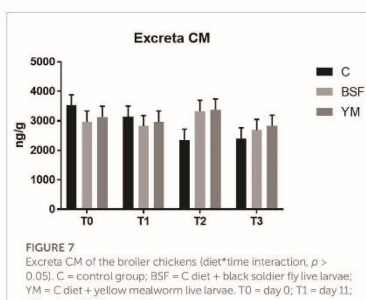

FIGURE 7
Excreta CM of the broiler chickens (diet*time interaction, $p > 0.05$). C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

to observe potential differences in broilers as well. Furthermore, the identification of very low mean values for both the HB and the FPD scores of the C birds (less than 1) suggested the presence of an health status of the legs that was already good independently of insect live larvae administration, thus, in turn, making more challenging to improve it.

Excreta corticosterone

The excreta CM of the broiler chickens of the present study were not affected by the administration of both the BSF and the YM live larvae as well. The measurement of excreta CM is a well-recognized, non-invasive method to quantify the stress response in poultry, which offers a more convenient and less disruptive alternative to traditional measures that require bird restraint and blood sampling (Weimer et al., 2018), and does not interrupt the animal behaviour (Hirschenhauser et al., 2012). However, it is fundamental to underline that many factors (such as age, sex, diet, metabolic rate, social status, early life experience, diurnal and seasonal variations, and differences in the hormone metabolism of individuals) may influence the excreta CM (Alm et al., 2014). Therefore, despite the positive, insect-related modulation in the bird behaviour herein highlighted, such variability could have probably hidden the potential differences in the excreta CM.

Conclusion

In conclusion, the administration of BSF and YM live larvae as environmental enrichment for broiler chickens was capable of positively influencing the bird welfare through the stimulation of foraging behaviour, increase in activity levels, and reduction of behaviours potentially attributable to frustration, without affecting the plumage status, the leg health, and the excreta CM. As behavioural outcomes suggested some preference of the broilers for YM live larvae, further research to confirm this preference is recommended. Considering that the administration of insect live larvae in the intensive farming may potentially lead to different outcomes—as a consequence of the high rearing densities and competitiveness among birds—additional research testing such innovative environmental enrichment in the commercial setup are strongly recommended.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the corresponding author upon reasonable request.

Ethics statement

The animal study was reviewed and approved by the Bioethical Committee of the University of Turin (Italy).

Author contributions

AS, IB, SBO, and LG designed the study. IB, SBO, MG, EF, and SD carried out the rearing work. MP, and DD provided the insect live larvae. IB and SBO gave the feathering scores. GC and SBO analysed the behavioural video recordings. MG, EF, and SD collected the excreta samples. SBO, EF, and EM analysed the excreta corticosterone. IB and SBO performed the statistical analysis. IB wrote the first draft of the manuscript. All the authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors MP and DD were employed by the company Entomics Biosystems.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Alm, M., Holm, L., Tauson, R., and Wall, H. (2014). Corticosterone metabolites in laying hen droppings-Effects of fiber enrichment, genotype, and daily variations. *Poult. Sci.* 93, 2615–2621. doi:10.3382/ps.2014-04193

Arnould, C., Bizeray, D., Faure, J. M., and Leterrier, C. (2004). Effects of the addition of sand and string to pens on use of space, activity, tarsal angulations and bone composition in broiler chickens. *Anim. Welf.* 13, 87–94. doi:10.1093/ps/84.12.1816

Baxter, M., and O'Connell, N. E. (2016). "Commercial comparison of potential dustbathing substrates for intensively farmed broilers," in Proceedings of the EAAP 67th Annual Meeting (Belfast, Ireland: Wageningen Academic Publishers), 398.

Bellezza Oddon, S., Biasato, I., Imarisio, A., Pipan, M., Dekleva, D., Colombino, E., et al. (2021). Black soldier fly and yellow mealworm live larvae for broiler chickens: Effects on bird performance and health status. *J. Anim. Physiol. Anim. Nutr.* 105, 10–18. doi:10.1111/jpn.13567

Bizeray, D., Estevez, I., Leterrier, C., and Faure, J. M. (2002). Effects of increasing environmental complexity on the physical activity of broiler chickens. *Appl. Anim. Behav. Sci.* 79, 27–41. doi:10.1016/S0168-1591(02)00083-7

Bokkers, E. A. (2004). *Behavioural motivations and abilities in broilers.* Wageningen, Netherlands: Wageningen University.

Bokkers, E. A. M., and Koene, P. (2003). Behaviour of fast- and slow growing broilers to 12 weeks of age and the physical consequences. *Appl. Anim. Behav. Sci.* 81, 59–72. doi:10.1016/S0168-1591(02)00251-4

Bokkers, E. A. M., and Koene, P. (2002). Sex and type of feed effects on motivation and ability to walk for a food reward in fast growing broilers. *Appl. Anim. Behav. Sci.* 79, 247–261. doi:10.1016/S0168-1591(02)00151-X

Bruce, D. W., McIlroy, S. G., and Goodall, E. A. (1990). Epidemiology of a contact dermatitis of broilers. *Avian Pathol.* 19, 523–537. doi:10.1080/03079459008418705

Bruce, E., Prescott, N., and Wathes, C. (2003). Preferred food rewards for laying hens in behavioural experiments. *Br. Poult. Sci.* 44, 345–349. doi:10.1080/0007166031000085490

Castellini, C., Mugnai, C., Moscati, L., Mattioli, S., Guarino Amato, M., Cartoni Mancinelli, A., et al. (2016). Adaptation to organic rearing system of eight different chicken genotypes: Behaviour, welfare and performance. *Italian J. Animal Sci.* 15, 37–46. doi:10.1080/1828051X.2015.1131893

Costa, P., Macchi, E., Valle, E., De Marco, M., Nucera, D. M., Gasco, L., et al. (2016). An association between feather damaging behavior and corticosterone metabolite excretion in captive African grey parrots (*Psittacus erithacus*). *Peer J.* 4, e2462. doi:10.7717/peerj.2462

Dawkins, M. S. (1989). Time budgets in red junglefowl as a baseline for the assessment of welfare in domestic fowl. *Appl. Anim. Behav. Sci.* 24, 77–80. doi:10.1016/0168-1591(89)90126-3

de Jong, I. C., and Gunnink, H. (2018). Effects of a commercial broiler enrichment programme with or without natural light on behaviour and other welfare indicators. *Animal* 13, 384–391. doi:10.1017/S1751731118001805

Delius, J. (1988). Preening and associated comfort behavior in birds. *Ann. N. Y. Acad. Sci.* 525, 40–55. doi:10.1111/j.1749-6632.1988.tb38594.x

Duncan, I. (1998). Behavior and behavioral needs. *Poult. Sci.* 77, 1766–1772. doi:10.1093/ps/77.12.1766

Duncan, I. J. H., and Wood-Gush, D. G. M. (1972). Thwarting of feeding behaviour in the domestic fowl. *Anim. Behav.* 20, 444–451. doi:10.1016/S0003-3472(72)80007-1

Ekstrand, C., Carpenter, T. E., Andersson, I., and Algers, B. (1998). Prevalence and control of foot-pad dermatitis in broilers in Sweden. *Br. Poult. Sci.* 39, 318–324. doi:10.1080/00071669888845

Friard, O., and Gamba, M. (2016). Boris: A free, versatile open-source event-logging software for video/audio coding and live observations. *Methods Ecol. Evol.* 7, 1325–1330. doi:10.1111/2041-210X.12584

Gasco, L., Biancarosa, I., and Liland, N. S. (2020). From waste to feed: A review of recent knowledge on insects as producers of protein and fat for animal feeds. *Curr. Opin. Green Sustain. Chem.* 23, 67–79. doi:10.1016/j.cogsc.2020.03.003

Giersberg, M. F., Molenaar, R., de Jong, I. C., Souza da Silva, C., van den Brand, H., Kemp, B., et al. (2021). Effects of hatching system on the welfare of broiler chickens in early and later life. *Poult. Sci.* 100, 100946. doi:10.1016/j.psj.2020.12.043

Hirschenhauser, K., Spreitzer, K., Lepschy, M., Kotrschal, K., and Möstl, E. (2012). Excreted corticosterone metabolites differ between two galliform species, Japanese Quail and Chicken, between sexes and between urine and faecal parts of droppings. *J. Ornithol.* 153, 1179–1188. doi:10.1007/s10336-012-0848-9

Iannetti, L., Romagnoli, S., Cotturone, G., and Podaliri Vulpiani, M. (2021). Animal welfare assessment in antibiotic-free and conventional broiler chicken. *Animals.* 11, 2822. doi:10.3390/ani11102822

Ipema, A. F., Bokkers, E. A. M., Gerrits, W. J. J., Kemp, B., and Bolhuis, J. E. (2021b). Providing live black soldier fly larvae (*Hermetia illucens*) improves welfare while maintaining performance of piglets post-weaning. *Sci. Rep.* 11, 7371. doi:10.1038/s41598-021-86765-3

Ipema, A. F., Bokkers, E. A. M., Gerrits, W. J. J., Kemp, B., and Bolhuis, J. E. (2020). Provisioning of live black soldier fly larvae (*Hermetia illucens*) benefits broiler activity and leg health in a frequency- and dose-dependent manner. *Appl. Anim. Behav. Sci.* 230, 105082. doi:10.1016/j.applanim.2020.105082

Ipema, A. F., Gerrits, W. J. J., Bokkers, E. A. M., Kemp, B., and Bolhuis, J. E. (2021a). Live black soldier fly larvae (*Hermetia illucens*) provisioning is a promising environmental enrichment for pigs as indicated by feed- and enrichment-preference tests. *Appl. Anim. Behav. Sci.* 244, 105481. doi:10.1016/j.applanim.2021.105481

Jacobs, L., Melick, S., Freeman, N., Garmyn, A., and Tuyttens, F. A. M. (2021). Broiler chicken behavior and activity are affected by novel flooring treatments. *Animals.* 11, 2841. doi:10.3390/ani11102841

Jordan, D., Stuhec, I., and Bessei, W. (2011). Effect of whole wheat and feed pellets distribution in the litter on broilers' activity and performance. *Arch. Geflugelkd.* 75, 98–103.

Kenny, E., Birkhead, T. R., and Green, J. P. (2017). Allopreening in birds is associated with parental cooperation over offspring care and stable pair bonds across years. *Behav. Ecol.* 28, 1142–1148. doi:10.1093/beheco/arx078

Lai, P. W., Liang, J. B., Hsia, L. C., Loh, T. C., and Ho, Y. W. (2010). Effects of varying dietary zinc levels and environmental temperatures on the growth performance, feathering score and feather mineral concentrations of broiler chicks. *Asian-Australas. J. Anim. Sci.* 23, 937–945. doi:10.5713/ajas.2010.90495

Li, G., Hui, X., Lin, F., and Zhao, Y. (2020). Developing and evaluating poultry preening behavior detectors via mask region-based convolutional neural network. *Animals.* 10, 1762. doi:10.3390/ani10101762

Lourenço da Silva, M. I., Almeida Paz, I. C. L., Chaves, G. H. C., Almeida, I. C. L., Ouros, C. C. D., Souza, S. R. L., et al. (2021). Behaviour and animal welfare indicators of broiler chickens housed in an enriched environment. *PLoS One* 16, e0256963. doi:10.1371/journal.pone.0256963

Louton, H., Piller, A., Bergmann, S., Erhard, M., Stracke, J., Spindler, B., et al. (2020). Histologically validated scoring system for the assessment of hock burn in broilers. *Avian Pathol.* 49, 230–242. doi:10.1080/03079457.2020.1712328

Mahmoud, U. T., Abdel-Rahman, M. A. M., Darwish, M. H. A., Applegate, T. J., and Cheng, H. (2015). Behavioural changes and feathering score in heat stressed broiler chickens fed diets containing different levels of propolis. *Appl. Anim. Behav. Sci.* 166, 98–105. doi:10.1016/j.applanim.2015.03.003

Martin, G., Sambraus, H. H., and Steiger, A. (2005). *Welfare of laying hens in europe – reports, analyses and conclusions*. Witzenhausen: Beratung Artgerechte Tierhaltung eV.

Newberry, R. C. (1995). Environmental enrichment: Increasing the biological relevance of captive environments. *Appl. Anim. Behav. Sci.* 44, 229–243. doi:10.1016/0168-1591(95)00616-Z

Opengart, K., Bilgili, S. F., Warren, G. L., Baker, K. T., Moore, J. D., and Dougherty, S. (2018). Incidence, severity, and relationship of broiler footpad lesions and gait scores of market-age broilers raised under commercial conditions in the southeastern United States. *J. Appl. Poult. Res.* 27, 424–432. doi:10.3382/japr/pfy002

Palme, R., Touma, C., Arias, N., Dominchin, M. F., and Lepschy, M. (2013). Steroid extraction: Get the best out of excreta samples. *Wien. Tierarztl. Monatsschr.* 100, 238–246.

Pichova, K., Nordgreen, J., Leterrier, C., Kostal, L., and Moe, R. O. (2016). The effects of food related environmental complexity on litter directed behaviour, fear and exploration of novel stimuli in young broiler chickens. *Appl. Anim. Behav. Sci.* 174, 83–89. doi:10.1016/j.applanim.2015.11.007

Ravi, H. K., Degrou, A., Costil, J., Trespeuch, C., Chemat, F., and Vian, M. A. (2020). Larvae mediated valorization of industrial, agriculture and food wastes: Biorefinery concept through bioconversion, processes, procedures, and products. *Processes* 8, 857. doi:10.3390/pr8070857

Reiter, K., and Bessei, W. (2009). Effect of locomotor activity on leg disorder in fattening chicken. *Berl. Munch. Tierarztl. Wochenschr.* 122, 264–270. doi:10.2376/0005-9366-122-264

Riber, A. B., van de Weerd, H. A., de Jong, I. C., and Steenfeldt, S. (2018). Review of environmental enrichment for broiler chickens. *Poult. Sci.* 97, 378–396. doi:10.3382/ps/pex344

Sevim, Ö., Ahsan, U., Tatlı, O., Kuter, E., Khamseh, E. K., Reman Temiz, A., et al. (2022). Effect of nano-selenium and high stocking density on performance, carcass yield, meat quality and feathering score of broilers. *Ank. Üniversitesi Veteriner Fakültesi Derg.* doi:10.33988/auvfd.838120

Shields, S. J., Garner, J. P., and Mench, J. A. (2005). Effect of sand and wood-shavings bedding on the behavior of broiler chickens. *Poult. Sci.* 84, 1816–1824. doi:10.1093/ps/84.12.1816

Sokołowicz, Z., Dykiel, M., Topczewska, J., Krawczyk, J., and Augustyńska-Prejsnar, A. (2020). The effect of the type of non-caged housing system, genotype and age on the behaviour of laying hens. *Animals.* 10, 2450. doi:10.3390/ani10122450

Star, L., Arsiwalla, T., Molist, F., Leushuis, R., Dalim, M., and Paul, A. (2020). Gradual provision of live black soldier fly (*Hermetia illucens*) larvae to older laying hens: Effect on production performance, egg quality, feather condition and behaviour. *Animals.* 10, 216. doi:10.3390/ani10020216

Tahamtani, F. M., Ivarsson, E., Wiklicky, V., Lalander, C., Wall, H., Rodenburg, T. B., et al. (2021). Feeding live black soldier fly larvae (*Hermetia illucens*) to laying hens: Effects on feed consumption, hen health, hen behaviour, and egg quality. *Poult. Sci.* 100, 101400. doi:10.1016/j.psj.2021.101400

Toghyani, M., Gheisari, A., Modaresi, M., Tabeidian, S. A., and Toghyani, M. (2010). Effect of different litter material on performance and behavior of broiler chickens. *Appl. Anim. Behav. Sci.* 122, 48–52. doi:10.1016/j.applanim.2009.11.008

van Hierden, Y. M., Korte, S. M., Ruesink, E. W., van Reenen, C. G., Engel, B., Koolhaas, J. M., et al. (2002). The development of feather pecking behaviour and targeting of pecking in chicks from a high and low feather pecking line of laying hens. *Appl. Anim. Behav. Sci.* 77, 183–196. doi:10.1016/S0168-1591(02)00046-1

Vasdal, G., Vas, J., Newberry, R. C., and Moe, R. O. (2019). Effects of environmental enrichment on activity and lameness in commercial broiler production. *J. Appl. Anim. Welf. Sci.* 22, 197–205. doi:10.1080/10888705.2018.1456339

Veldkamp, T., and van Niekerk, T. G. C. M. (2019). Live black soldier fly larvae (*Hermetia illucens*) for Turkey poults. *J. Insects Food Feed* 5, 301–311. doi:10.3920/JIFF2018.0031

Webster, A. B., and Hurnik, J. F. (1990). An ethogram of White Leghorn-Type hens in battery cages. *Can. J. Anim. Sci.* 70, 751–760. doi:10.4141/cjas90-094

Weimer, S. L., Wideman, R. F., Scanes, C. G., Mauromoustakos, A., Christensen, K. D., and Vizzier-Thaxton, Y. (2018). An evaluation of methods for measuring stress in broiler chickens. *Poult. Sci.* 97, 3381–3389. doi:10.3382/ps/pey204

10. Discussion and Conclusion

The scientific contributions of the present PhD thesis follow the two essential aspects of the application of insect rearing to the circular economy model: the maximisation of the use of waste-based substrates and the inclusion or addition of insect-derived products in animal feed production. Waste optimisation can be achieved through the determination of the nutritional requirements and the substrate formulation (1st aim), while the feasibility of use of insect meal and live larvae in livestock diets can be determined by testing such products through experimental trials (2nd aim).

Insect rearing

As far as insect farming is concerned, the two scientific publications on the determination of the nutrient requirements of BSF are among the first works published related to this research topic. Considering that the semi-purified diets used for the determination of the protein and lipid BSF requirements did not affect the survival rate, it is possible to hypothesise that they are suitable for the inclusion of larvae. Since the diets are composed by semi-purified ingredients that are meals, the substrate texture has some negative aspects. Due to this reason, it is necessary to cover the rearing boxes with a lid – which is a condition that is not applicable to an industrial rearing. Therefore, the improvement of the physical characteristics of the semi-purified diets is important to make the optimal trial settings. Among the protein levels that were tested, 16% of crude protein on a dry matter (DM) basis seemed to be the best percentage for the entire larval stage. Instead, considering the larva days of age - up to 14 days' old – protein levels equal to 10%, on a DM basis, showed a negative effect on growth, while this difference was no longer noticeable in the later

stages - 18 days and prepupa. The same trend was observed during the different instar development stages of the larvae for the lipid levels. On the basis of these outcomes, it is possible to hypothesise a greater lipid requirement (4.5% on a DM basis) during the first stages of growth, which becomes less evident in the later larval instars. Moreover, the size and the development time were negatively affected by a lipid level equal to 1% on a DM basis. Further research is needed to evaluate a wide range of nutrient levels and also their quality (amino acid and fatty acid profiles). Moreover, given the lack of scientific information on the physiology, metabolism and digestibility of BSF, it is currently difficult to understand what factors influenced the trial results. For this reason, in addition to the nutritional requirements, further studies on the texture and digestibility of substrates are necessary to create a clearer scientific framework and facilitate the basic choice of a farm: the breeding substrate.

Insect meal in aquaculture

The effects of different BSF defatted meals included as substitutes of fishmeal in low fish-based diets for rainbow trout have been discussed in two scientific contributions. The first trial was conducted with lower inclusion levels (3 – 6 – 9 – 12 and 15%) than the second (8 – 16 – 32%). Both trials showed that increasing the BSF inclusion levels had no effect on the growth performance, the dry matter, protein, ether extract and gross energy apparent digestibility coefficients, the histomorphological features of the animals or the physical and chemical composition of the fillets, although the fatty acid profile was an exception. In particular, a decrease in PUFA and an increase in the total SFA and MUFA were observed as the BSF inclusion was increased. The results obtained agree with Renna et al. (2017), in which the inclusion of 20% of defatted BSF meal did not affect all the above reported parameters. On the other hand, the authors

observed reduced protein and ether extract apparent digestibility coefficients with the 40% inclusion level (HI40) and a large difference of the fillet fatty acid profile between the control and the HI40 treatments (Renna et al., 2017). On the basis of the above reported outcomes, and since the use of insects can reduce environmental impacts and support a sustainable aquaculture production, BSF defatted meal can be considered a promising and suitable ingredient for rainbow trout diets.

Live larvae as an environmental enrichment practice in poultry

The two scientific publications regarding the supplementation of broiler chicken diets with live BSF and YM larvae covered different but equally important aspects, such as the growth performance, the health status and animal welfare, which need to be considered to fully evaluate the use of live larvae as an environmental enrichment practice. The growth performance of the birds was not negatively affected by the administration of 5% of the daily feed intake of BSF and YM larvae. Furthermore, the birds administered TM larvae showed a better overall FCR than the other groups. The larva ingestion time variable revelated that the broiler chickens were eager to eat live larvae. Moreover, birds spent less time ingesting YM larvae than BSF larvae. This result could be explained as a preference of the birds for larvae, probably due to their movement, colour and flavour. As regard the health status (histomorphological investigations, haematological and serum parameters), no relevant differences were observed between the control and the live insect larva groups. Finally, many different behaviour patterns were influenced by the administration of live larvae. In particular, in agreement with Ipema et al. (2020), the foraging behaviour and activity were stimulated by such an environmental enrichment. The plumage status, the leg health and the excreta corticosterone were instead comparable between treatments. In

conclusion, the administration of live larvae as an environmental enrichment practice seems to positively affect animal welfare, and even result in some performance improvements linked to the used insect species.

11. Overall conclusion

The nutritional levels determined until now for the BSF are general guidelines to be deepened. Specifically, the assessment of the limiting factors (amino acids, vitamins, minerals) will be an important step for the research. At the same time, this research issue is closely linked to other lines of research such as genetics and insect digestibility of nutrients and - for this reason - it needs to follow their path. The research applied on the use of insect-derived products is advanced when compared to the insect rearing, but it still needs to answer several questions – such as the identification of an optimal method for the meal processing and, consequently, a maximum inclusion value for the various meal (whole/defatted; different species). Finally, considering the use of live larvae as environmental enrichment in poultry farm, they seem to have a positive effect on the animal welfare. Further researches will be needed in order to assess the feasibility of this type of production, such as storage method and the safety risks.

12. Other contributions

Gariglio M., Dabbou S., Gai F., Trocino A., Xiccato G., Holodova M., Gresakova L., Nery J., **Bellezza Oddon S.**, Biasato I., Gasco L., Schiavone A., 2021. *Black soldier fly larva in Muscovy duck diets: effects on duck growth, carcass property, and meat quality.* Poultry Science. 10.1016/j.psj.2021.101303

Colombino E., Biasato I., Ferrocino I., **Bellezza Oddon S.**, Caimi C., Gariglio M., Dabbou S., Caramori M., Battisti E., Zanet S., Ferroglio E., Cocolin L., Gasco L., Schiavone A., Capucchio M.T., 2021. *Effect of Insect Live Larvae as Environmental Enrichment on Poultry Gut Health: Gut Mucin Composition, Microbiota and Local Immune Response Evaluation*. Animals, 10.3390/ani11102819

Jucker C., Belluco S., **Bellezza Oddon S.**, Ricci A., Bonizzi L., Lupi D., Savoldelli S., Biasato I., Caimi C., Mascaretti A., Gasco L., 2021. *Impact of some local organic by-products on Acheta domesticus growth and meal production*. Journal of Insects as Food and Feed. 10.3920/JIFF2021.0121

Palomba A., Melis R., Biosa G., Braca A., Pisanu S., Ghisaura S., Caimi C., Biasato I., **Bellezza Oddon S.**, Laura G., Terova G., Moroni F., 2022. *On the compatibility of fish meal replacements in aquafeeds for rainbow trout. a combined metabolomic, proteomic and histological study.* Frontiers in Physiology. 10.3389/fphys.2022.920289

Gasco L., Caimi C., Trocino A., Lussiana C., **Bellezza Oddon S.**, Malfatto V., Anedda R., Serra G., Biasato I., Schiavone A., Gai F., Renna M., 2022. *Characterization of the digestibility of defatted insect meals for rainbow trout feeding.* Journal of Insects as Food and Feed. 10.3920/JIFF2021.0160

13. References

Barragán-Fonseca, K. B., Gort, G., Dicke, M., & van Loon, J. J. (2019). *Effects of dietary protein and carbohydrate on life-history traits and body protein and fat contents of the black soldier fly Hermetia illucens.* Physiological Entomology, 44(2), 148-159.

Barragán-Fonseca, K. B., Gort, G., Dicke, M., & Van Loon, J. J. A. (2021). *Nutritional plasticity of the black soldier fly (Hermetia illucens) in response to artificial diets varying in protein and carbohydrate concentrations*. Journal of Insects as Food and Feed, 7(1), 51-61.

Bhadauria, P., & Bhanja, S. K. (2017). *Practicing behavioural observations for assessment of welfare in poultry.* ICAR-CARI, Izzatnagar.

Bilcik, B., & Keeling, L. J. (1999). *Changes in feather condition in relation to feather pecking and aggressive behaviour in laying hens*. British poultry science, 40(4), 444-451.

Blokhuis, H. J. (1986). *Feather-pecking in poultry: its relation with ground-pecking.* Applied Animal Behaviour Science, 16(1), 63-67.

Bosch, G., Oonincx, D. G. A. B., Jordan, H. R., Zhang, J., Van Loon, J. J. A., Van Huis, A., & Tomberlin, J. K. (2020). *Standardisation of quantitative resource conversion studies with black soldier fly larvae*. Journal of Insects as Food and Feed, 6(2), 95-109.

Brambell, R., 1965. *Report of the technical committee to enquire into the welfare of animals kept under intensive livestock husbandry systems.* Her Majesty's Stationery Office. London, 85 pp.

Cammack, J. A., & Tomberlin, J. K. (2017). *The impact of diet protein and carbohydrate on select life-history traits of the black soldier fly Hermetia illucens* (L.)(Diptera: Stratiomyidae). Insects, 8(2), 56.

Cappellozza, L., Cappellozza, S., Saviane, A., & Sbrenna, G. (2005). *Artificial diet rearing system for the silkworm Bombyx mori (Lepidoptera: Bombycidae): effect of vitamin C deprivation on larval growth and cocoon production*. Applied Entomology and Zoology, 40(3), 405-412.

Cardinaletti, G., Di Marco, P., Daniso, E., Messina, M., Donadelli, V., Finoia, M. G., Petochi T., Fava F., Faccenda F., Contò M., Cerri R., Volpatti D., Bulfon C., Mandich A., Longobardi A., Marino G., Pulido-Rodriguez L. F., Parisi G., Tibaldi, E., 2022. *Growth and welfare of rainbow trout (Oncorhynchus mykiss) in response to graded levels of insect and poultry by-product meals in fishmeal-free diets.* Animals, 12(13), 1698.

Chapman, R. F., & Chapman, R. F. (2012). *The insects: structure and function.* Cambridge university press.

Cohn, Z., Latty, T., & Abbas, A. (2022). *Understanding dietary carbohydrates in black soldier fly larvae treatment of organic waste in the circular economy*. Waste Management, 137, 9-19.

Cortés M., Tomás-Almenar, C., 2022. *Fishmeal dietary replacement up to 50%: a comparative study of two insect meals for rainbow trout (Oncorhynchus mykiss)*. Animals, 12(2), 179.

Diener, S., Zurbrügg, C., Gutiérrez, F. R., Nguyen, D. H., Morel, A., Koottatep, T., & Tockner, K. (2011). *Black soldier fly larvae for organic waste treatment-prospects and constraints*. Proceedings of the WasteSafe, 2, 13-15.

EUMOFA, (2021). *Fishmeal and fish oil: Production and trade flows in the EU*. European Market Observatory for fisheries and aquaculture. Brussels.

Ferrante, V. (2009). *Welfare issues of modern laying hen farming*. Italian journal of animal science, 8(sup1), 175-189.

Fischer, H., Romano, N., Renukdas, N., Kumar, V., Sinha, A. K., 2022. *Comparing black soldier fly (Hermetia illucens) larvae versus prepupae in the diets of largemouth bass, Micropterus salmoides: Effects on their growth, biochemical composition, histopathology, and gene expression*. Aquaculture, 546, 737323.

Fraser, D., Weary, D. M., Pajor, E. A., & Milligan, B. N. (1997). *A scientific conception of animal welfare that reflects ethical concerns.* Animal welfare, 6: 187-205.

Georgescu, B., Struţi, D., Papuc, T., Cighi, V., & Boaru, A. (2021). *Effect of the energy content of diets on the development and quality of the fat reserves of larvae and reproduction of adults of the black soldier fly, Hermetia illucens (Diptera: Stratiomyidae)*. European Journal of Entomology, 118, 297-306.

Gold, M., Cassar, C. M., Zurbrügg, C., Kreuzer, M., Boulos, S., Diener, S., & Mathys, A. (2020). *Biowaste treatment with black soldier fly larvae: Increasing performance through the formulation of biowastes based on protein and carbohydrates*. Waste Management, 102, 319-329.

Hoc, B., Tomson, T., Malumba, P., Blecker, C., Jijakli, M. H., Purcaro, G., Francis F., Megido, R. C., 2021. *Production of rainbow trout (Oncorhynchus mykiss) using black soldier fly (Hermetia illucens) prepupae-based formulations with differentiated fatty acid profiles*. Science of the Total Environment, 794, 148647.

Hua, K. (2021). *A meta-analysis of the effects of replacing fish meals with insect meals on growth performance of fish*. Aquaculture, 530, 735732.

International Buyers' Guide (2015). *U.S. Soy*. (https://ussec.org/wp-content/uploads/2015/10/buyers-guide.pdf)

Ipema, A. F., Gerrits, W. J., Bokkers, E. A., Kemp, B., & Bolhuis, J. E. (2020). *Provisioning of live black soldier fly larvae (Hermetia illucens) benefits broiler activity and leg health in a frequency-and dose-dependent manner*. Applied Animal Behaviour Science, 230, 105082.

IPIFF (2022). *Guide on Good Hygiene Practices* (https://ipiff.org/good-hygiene-practices/)

Józefiak, A., Nogales-Mérida, S., Mikołajczak, Z., Rawski, M., Kierończyk, B., & Mazurkiewicz, J. (2019). *The utilization of full-fat insect meal in rainbow trout nutrition: the effects on growth performance, intestinal microbiota and gastrointestinal tract histomorphology*. Annals of Animal Science, 19(3), 747-765.

Liland, N. S., Araujo, P., Xu, X. X., Lock, E. J., Radhakrishnan, G., Prabhu, A. J. P., & Belghit, I. (2021). *A meta-analysis on the nutritional value of insects in aquafeeds*. Journal of Insects as Food and Feed, 7(5), 743-759.

Lorimor, J., Fulhage, C., Zhang, R., Funk, T., Sheffield, R., Sheppard, D. C., & Newton, G. L. (2006). *Manure management strategies and technologies.*

Lund, V., Coleman, G., Gunnarsson, S., Appleby, M. C., & Karkinen, K. (2006). *Animal welfare science—Working at the interface between the natural and social sciences*. Applied Animal Behaviour Science, 97(1), 37-49.

Makkar, H. P., Tran, G., Heuzé, V., & Ankers, P. (2014). *State-of-the-art on use of insects as animal feed*. Animal feed science and technology, 197, 1-33.

McCulloch, S. P. (2013). *A critique of FAWC's five freedoms as a framework for the analysis of animal welfare*. Journal of agricultural and environmental ethics, 26(5), 959-975.

Melenchón, F., de Mercado, E., Pula, H. J., Cardenete, G., Barroso, F. G., Fabrikov, D., Lourenço H.M., Pessoa M.F., Lagos L., Weththasinghe P., Michalk, D. L., Kemp, D. R., Badgery, W. B., Wu, J., Zhang, Y., & Thomassin, P. J. (2019). *Sustainability and future food security—a global perspective for livestock production*. Land Degradation & Development, 30(5), 561-573.

Newton, L. A. R. R. Y., Sheppard, C. R. A. I. G., Watson, D. W., Burtle, G. A. R. Y., & Dove, R. O. B. E. R. T. (2005). *Using the black soldier fly, Hermetia illucens, as a value-added tool for the management of swine manure.* Animal and Poultry Waste Management Center, North Carolina State University, Raleigh, NC, 17(2005), 18.

Newberry, R. C. (1995). *Environmental enrichment: Increasing the biological relevance of captive environments*. Applied Animal Behaviour Science, 44(2-4), 229-243.

Ocha, I. M., Ujah, M. O., Adeniyi, K. A., Ochuole, J. O., & Yahaya, A. W. (2022). *the contribution of insects to sustainable food security, livelihoods and environment: a review*. WATARI Multi-disciplinary Journal of Science, Technology and Mathematics Education, 6(1), 100-113.

Oonincx, D. G., van Broekhoven, S., van Huis, A., & van Loon, J. J. (2019). *Feed conversion, survival and development, and composition of four insect species on diets composed of food by-products*. PLoS One, 14(10), e0222043.

Pauly, D., Zeller, D. & Palomares, M.L.D. (2020). *Sea Around Us Concepts, Design and Data* (seaaroundus.org)

Renna, M., Schiavone, A., Gai, F., Dabbou, S., Lussiana, C., Malfatto, V., ... & Gasco, L. (2017). Evaluation of the suitability of a partially defatted black soldier fly (Hermetia illucens L.) larvae meal as ingredient for rainbow trout (Oncorhynchus mykiss Walbaum) diets. Journal of animal science and biotechnology, 8(1), 1-13.

Reyes, M., Rodríguez, M., Montes, J., Barroso, F. G., Fabrikov, D., Morote, E., Sánchez-Muros, M. J., 2020. *Nutritional and growth effect of insect meal inclusion on seabass (Dicentrarchuss labrax) feeds*. Fishes, 5(2), 16.

Riber, A. B., Van de Weerd, H. A., De Jong, I. C., & Steenfeldt, S. (2018). *Review of environmental enrichment for broiler chickens*. Poultry science, 97(2), 378-396.

Ritchie, H. & Roser, M. (2022). *Soy*. Retrieved on 30 September 2022, from https://ourworldindata.org/soy.

Siddiqui, S. A., Ristow, B., Rahayu, T., Putra, N. S., Yuwono, N. W., Mategeko, B., ... & Nagdalian, A. (2022). *Black soldier fly larvae (BSFL) and their affinity for organic waste processing*. Waste Management, 140, 1-13.

Singh, A., & Kumari, K. (2019). *An inclusive approach for organic waste treatment and valorisation using Black Soldier Fly larvae: A review*. Journal of Environmental Management, 251, 109569.

Sogbesan, O. A., Isa, J., Madu, C. T., Koyenme, J. E., Adewole, A. M., & Dan Ologe, I. A. (2007). *Comparison of maggot culture as aquafeed in sustainable aquaculture using different organic wastes*.

Star, L., Arsiwalla, T., Molist, F., Leushuis, R., Dalim, M., & Paul, A. (2020). *Gradual provision of live black soldier fly (Hermetia illucens) larvae to older laying hens: effect on production performance, egg quality, feather condition and behavior*. Animals, 10(2), 216.

Tahamtani, F. M., Ivarsson, E., Wiklicky, V., Lalander, C., Wall, H., Rodenburg, T. B., ... & Hernandez, C. E. (2021). *Feeding live Black Soldier Fly larvae (Hermetia illucens) to laying hens: effects on feed consumption, hen health, hen behavior, and egg quality.* Poultry science, 100(10), 101400.

Tran, H. Q., Nguyen, T. T., Prokešová, M., Gebauer, T., Doan, H. V., Stejskal, V., 2022. Systematic review and meta-analysis of production performance of aquaculture species fed dietary insect meals. Reviews in Aquaculture, 14, 1637–1655.

Tschirner, M., & Simon, A. (2015). Influence of different growing substrates and processing on the nutrient composition of black soldier fly larvae destined for animal feed. Journal of insects as food and feed, 1(4), 249-259.

van Huis, A. (2022). *Cultural significance of locusts, grasshoppers, and crickets in sub-Saharan Africa*. Journal of Ethnobiology and Ethnomedicine, 18(1), 1-19.

Wachira, M. N., Osuga, I. M., Munguti, J. M., Ambula, M. K., Subramanian, S., Tanga, C. M., 2021. Efficiency and improved profitability of insect-based aquafeeds for farming Nile Tilapia fish (Oreochromis niloticus L.). Animals, 11(9), 2599.

Weaver, D. K., & McFarlane, J. E. (1990). *The effect of larval density on growth and development of Tenebrio molitor*. Journal of Insect Physiology, 36(7), 531-536.

Weththasinghe, P., Lagos, L., Cortés, M., Hansen, J. Ø., Øverland, M., 2021. Dietary inclusion of black soldier fly (Hermetia illucens) larvae meal and paste improved gut health but had minor effects on skin mucus proteome and immune response in Atlantic salmon (Salmo salar). Frontiers in immunology, 12, 599530.

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