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**Insect meal and live larvae as innovative feedstuff in ducks**

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*Vorrei dedicare questa tesi alla mia famiglia.*

*Alla mia mamma, che mi è sempre stata vicina in questi anni e mi ha trasmesso la sua forza;  
A mio fratello, che ha sempre avuto un momento da dedicarmi e conosce a memoria il mio lavoro;  
A mio zio, che ha sempre avuto una parola di incoraggiamento per me.*

*A tutti voi, grazie.*

# Abbreviations

|                                                        |                                                |
|--------------------------------------------------------|------------------------------------------------|
| AA: amino acids                                        | HC: hot carcass                                |
| ADF: acid detergent fiber                              | HE: Haematoxylin & Eosin                       |
| ADG: average daily gain                                | HI: <i>Hermetia illucens</i>                   |
| AI: atherogenicity index                               | LM: leg muscle                                 |
| ALP: alkaline phosphatase                              | LW: live weight                                |
| ALT: alanino-aminotransferase                          | MD: <i>Musca domestica</i>                     |
| AME: apparent metabolizable energy                     | MDA: malondialdehyde                           |
| AMEn: apparent metabolizable energy nitrogen corrected | ME: metabolizable energy                       |
| AST: aspartate-aminotransferase                        | MG: methylglyoxal                              |
| ATTDC: apparent total tract digestibility coefficients | MUFA: monounsaturated fatty acid               |
| BM: breast muscle                                      | NDF: neutral detergent fiber                   |
| BW: body weight                                        | OM: organic matter                             |
| CC: chilled carcass                                    | OTUs: Operational Taxonomic Units              |
| Cd: crypt depth                                        | PAS: Periodic acid-Schiff                      |
| CP: crude protein                                      | PCA: Principal component analysis              |
| DFI: daily feed intake                                 | PUFA: polyunsaturated fatty acid               |
| DM: dry matter                                         | SB: Sudan Black                                |
| EE: ether extract                                      | SEM: standard error of the means               |
| Exp 1: Experiment 1                                    | SFA: saturated fatty acid                      |
| Exp 2: Experiment 2                                    | SW: slaughtering weight                        |
| FA: fatty acid                                         | TAS: total antioxidant status                  |
| FAME: fatty acid methyl ester                          | TBARS: thiobarbituric acid reactive substances |
| FCR: feed conversion ratio                             | TI: thrombogenicity index                      |
| FDR: false discovery rate                              | TM: <i>Tenebrio molitor</i>                    |
| GGT: gamma glutamyl transferase                        | UA: uric acid                                  |
| GHG: greenhouse gasses                                 | Vh/Cd: villus height-to-crypt depth            |
| GPx: glutathione peroxidase                            | Vh: villus height                              |
| H/L: heterophils to lymphocytes ratio                  | WBSF: Warner-Bratzler shear force              |
| Hb: haemoglobin                                        |                                                |

# **Chapter 1**

## ***Introduction***

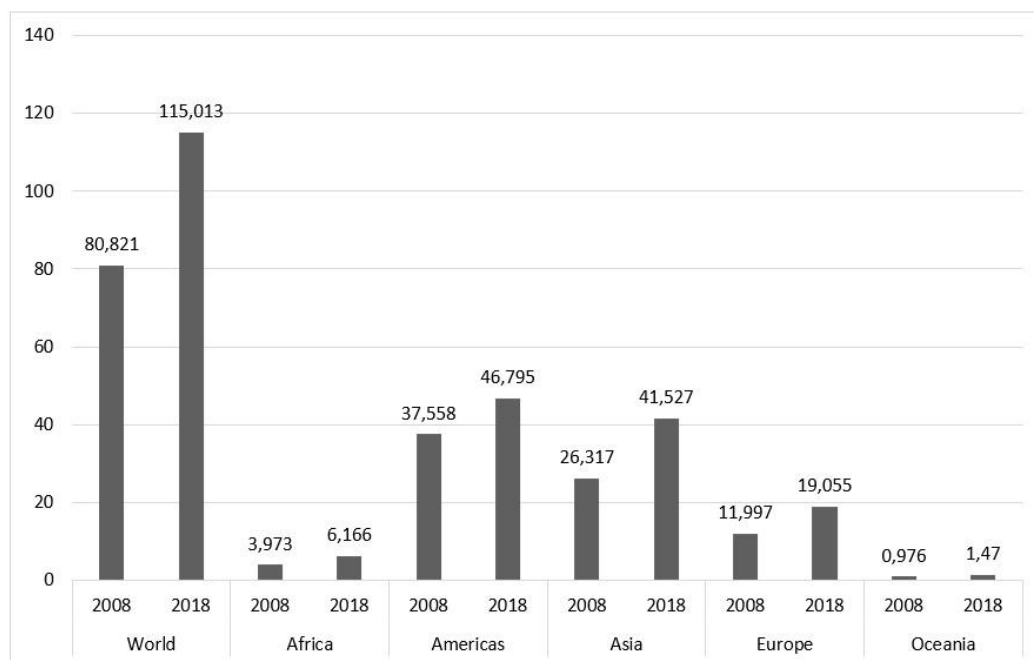
# 1. Introduction

In September 2015, the General Assembly of the United Nations has drafted a plan entitled 'the 2030 Agenda for Sustainable Development', articulated in goals focused to "shift the world on to a sustainable and resilient path" (United Nations, 2015; Dicke, 2018). One of the identified objectives is related to the improvement of food security and nutrition, supporting a sustainable agriculture in an optic of circular production of food for humans (United Nations, 2015; Dicke, 2018). Indeed, in a context of increasing world population, the future global demand of agricultural products will rapidly increase, with an expansion of 1.1% per year from 2005/2007 to 2050 (Alexandratos and Bruisma, 2012). The growing of food per capita consumption, together with changes in diet habits, will determine an increase in livestock consumption (more than 75% by 2050, at a global level), especially in developing countries (such as China and Brazil). This phenomenon is the so-called "livestock revolution", that is the shift of diet toward animal products, with a rapid increase in meat consumption (Delgado et al., 2001; Alexandratos and Bruisma, 2012; van Huis and Oonincx, 2017). This trend brings with it many critical issues, especially from an environmental point of view. It has been estimated that agriculture is responsible of the 80% of worldwide deforestation, especially in Latin America. Particularly, the land use is strictly linked to the livestock feed production, that currently occupy the 80% for animal grazing or the production of livestock feed and fodder (Alexandratos and Bruisma, 2012; FAO, 2017; van Huis and Oonincx, 2017). One of the largest crops in the world is soybean, destined mainly to be processed into soy oil and soy cake for animal feed as vegetable protein source (87% of the global produced soybean) (Fraanje and Garnett, 2020). On the other hand, there are other environmental implications linked to the expansion of livestock production that must be taken in account, that are water pollution and greenhouse gasses (GHG) emissions (FAO, 2017; Mateo-Sagasta et al., 2017; van Huis and Oonincx, 2017). These problems associated to the intensive development of agriculture, and livestock in particular, will determine the necessity to discover and evaluate new and alternative protein sources for animal feeding, in order to relieve this environmental pressure. In order to promote a sustainable food consumption, production, and management of natural resources a circular economy approach should be used, promoting the waste reduction, together with their recycling and reuse (United Nations, 2015; Dicke, 2018). From this point of view, the use of insects as feed raw materials for livestock species could be one of the solutions. The possibility to growth insects on organic waste perfectly suit in a circular economy approach, transforming waste into high value feed sources. Moreover, other additional benefits are related to the use of insects as feed, such as positive effects on health and welfare of livestock, also reducing the antibiotic use in livestock production (Dicke, 2018).

## 2. Poultry trends

In the context of growing consumption of animal products, poultry sector is the one that is having the highest development in the recent years. This increase in poultry meat consumption is related to several factors, one of them is related to the absence of religious taboos related to poultry meat. Moreover, the consumption of poultry meat is increasingly growing worldwide due to the price and health factors, and it is expected that poultry meat will be the most consumed meat by 2022 (expected increase of 1.8% per year by 2050) (Alexandratos and Bruisma, 2012; Henchion et al., 2014). The FAO (2020) estimated that the poultry meat production will reach 136.8 million tonnes at the end of 2020, with an increase of 7.5% compared to 2018, and the increment will be higher in China, EU, UK, and Brazil. The poultry sector includes several species, such as chicken, duck, turkey, and geese. The main bred poultry species worldwide is chicken, that accounted about 115 million tonnes in 2018 (FAOSTAT, 2020) (Figure 1). The American continent is the bigger producer of chicken meat at a global level, with the major production mainly located in South America (FAOSTAT, 2020).

**Figure 1.** World and by region chicken meat production between 2008-2018 (million tonnes)  
(Source: FAOSTAT, 2020).



Among the other poultry species raised for meat production, duck breeding is the second in terms of live animals produced worldwide, with a total of 1.12 billion ducks (live animals) reared in 2018 (FAOSTAT, 2020). In the last twenty years the global production of duck meat almost doubled, moving

from 2.6 million tonnes in 1998 to 4.5 million tonnes in 2018. In 2018, the main producer of duck meat worldwide was Asia, with a total of 3.7 million tonnes, and China resulted to be the main producer, accounting for 3 million tonnes in 2018. While in some areas of the world the duck rearing represents an important economic production, in other regions the production and consumption of duck meat is very limited, probably related to a lower promotion of this type of meat for human consumption (Aronal et al., 2012). Indeed, nevertheless Europe is the second main producer of duck meat worldwide, its production accounted approximately for 0.5 million tonnes in 2018 (FAOSTAT, 2020).

In Europe, the main producer is France accounting for 47.31% of the total European production, followed by Hungary, Germany, and Poland that represent the 17.99, 7.12, and 4.69% in 2018, respectively (FAOSTAT, 2020). In Italy, duck meat sector has a little relevance compared to other poultry production. Indeed, the total amount of slaughtered ducks in Italy is decreasing, passing from 1.48 thousand birds in 2018 to 0.80 thousand birds in 2019 (ISTAT, 2020).

Despite this, duck farming has a big potential, since ducks are one of the fastest producers of animal proteins and could supply the future nutritional needs of the growing world population (Adeola, 2006).

### 3. Welfare and Behaviour

Animal welfare is a multidimensional issue which comprise scientific, ethical, economic, and political dimensions (Carenzi and Verga, 2009). An interdisciplinary approach is necessary in order to evaluate animal welfare, since it comprehends animals' good health, comfort, and expression of their natural behaviours (Botreau et al., 2007; Carenzi and Verga, 2009). Indeed, the evaluation and determination of animal welfare requires synergy between different research disciplines, such as biological and veterinary sciences, as well as ethology (Carenzi and Verga, 2009). The attention to animal welfare, with particular reference to the farmed animals, makes its appearance in 1965, when the Brambel Report (1965) listed the so-called "five freedoms" necessary to guarantee animals' well-being. The "five freedoms", widely applied in EU regulations and certification schemes (Botreau et al., 2007), are: 1) freedom from hunger and thirst; 2) freedom from discomfort; 3) freedom from pain, injury, and disease; 4) freedom to express normal behaviour; and 5) freedom from fear and distress.

More recently, the European project named Welfare Quality<sup>®</sup> (2009) has set criteria and sub criteria necessary to determine and guarantee animal welfare: 1) good feeding (absence of prolonged hunger and thirst); 2) good housing (comfort around resting, thermal comfort, and ease movement); 3) Good health (absence of injuries, disease, and pain from management procedures); and 4) appropriate behaviour (expression of social and other behaviours, good human-animal relationship, and positive emotional state) (Welfare Quality<sup>®</sup>, 2009). The necessity to assess animal welfare has become particularly significant in the recent years, due to the even more interest by the consumers about the living conditions of farmed animals (Botreau et al., 2007; Broom, 2017). Indeed, an increase in citizen interest concerning animal welfare has been observed in Europe from 2006 to 2015 (34 and 57%, respectively; Alonso et al., 2020). One of the main welfare topics is related to animals' confinement systems, become even more necessary in a context of increasing meat production (Fraser, 2008). Indeed, a survey carried out on European consumers (European Commission, 2005) indicated different factors important in terms of animal welfare. The first in order of importance was the "space allowance". Moreover, other important aspects were related to the possibility to access to outdoor areas, the absence of movement restrictions and the possibility for the animals to perform their natural behaviours (Martelli, 2009).

Currently, The United Nation Committee on World Food Security included in its "Proposed draft recommendations on sustainable agricultural development for food security and nutrition including the role of livestock" a Recommendation named "Animal health and welfare": "Improve animal welfare delivering on the five freedoms and related OIE standards and principles, including through capacity building programs, and supporting voluntary actions in the livestock sector to improve animal welfare" (United Nations, 2016; Alonso et al., 2020). In Europe, the Council Directive 98/58/EC regarding the "protection of animals kept for farming purposes" defines the minimum standards for the protection of

all farmed animals in terms of protection against pain or injury, physiological and ethological needs (European Commission, 1998). Moreover, specific European Directives have been drafted in order to establish the protection of the different farmed animal species, then transposed in each European State. Regarding poultry species, two Legislative Decree have been adopted in Italy: Legislative Decree n. 181 of 2010 (broiler chickens) and Legislative Decree 267 of 2003 (laying hens), regulating mainly the stocking density and the housing conditions. However, regarding duck rearing, no legislations on keeping conditions and management have been enacted in Europe (Broom, 2017).

The intensification of poultry production brings with it many animal welfare problems, such as damaging behaviour, injuries of specific body parts, various kinds of diseases, and the increased mortality rate (Relić et al., 2019). As reported above, welfare problems mainly derive from the impossibility for animals to express their natural behaviour, that can evolve in frustration, abnormal behaviours, and injuries (Relić et al., 2019). Regarding domestic ducks, their natural behavioural pattern does not differ so far from that of their progenitor, being good walkers, flyers, and swimmer (Rodenburg et al., 2005; Relić et al., 2019). Moreover, ducks spend a lot of time performing preening behaviours, with body shaking movements and cleaning (Rodenburg et al., 2005). In case of ducks' confinement, high stocking densities and/or absence of litter abnormal behaviours may occur, that could damage the birds itself or its conspecific (Relić et al., 2019). One of the main behavioural problems in ducks reared for meat production is injurious feather pecking and cannibalism (Rodenburg et al., 2005; Relić et al., 2019). Duck that exhibits injurious feather pecking to its conspecific manifests hard, fast, and singular pecks on the tail, back, vent, and neck, and this behaviour could result in cannibalistic phenomenon, with consequent serious bleeding and eventually determine the death of the injured bird (Relić et al., 2019). Nowadays, the commonly procedure performed in order to reduce injurious feather pecking and cannibalism is the bill-trimming, but this procedure determine a reduction in animal welfare (Rodenburg et al., 2005). Provide a more stimulating environment could help to reduce aggressive phenomena, for example with water bath or feed (straw or hay) supply or allowing outdoor access to the birds (Knierim et al., 2002; Relić et al., 2019). Indeed, an outdoor space with open water supplementation, as well as low stocking densities (6.3 birds/m<sup>2</sup>) significantly reduce feather pecking in Muscovy ducks (Bilasing et al., 1992; Klemm et al., 1992). To conclude, it is well recognised that allow the possibility to the animals to express their natural behavioural patterns can improve their welfare. Observing and understanding birds' behaviour can be a good indicator of their well-being.

## 4. Domestic duck

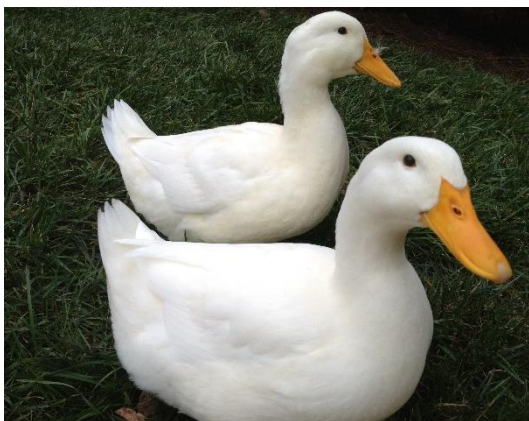
The domestic duck could be classified into two categories, that are the common duck (*Anas platyrhynchos*) and the Muscovy duck (*Cairina moschata*) (Table 1).

**Table 1.** Domestic duck classification (Crawford, 1990)

|             |                           |                         |
|-------------|---------------------------|-------------------------|
| Order       | Anseriformes              |                         |
| Family      | Anatidae                  |                         |
| Sub-family  | Anatinae                  |                         |
| Genus       | Anas                      | Cairinini               |
| Species     | <i>Anas platyrhynchos</i> | <i>Cairina moschata</i> |
| Common name | Mallard duck              | Muscovy duck            |

The main duck species used for meat production are Pekin (*A. platyrhynchos domesticus*), Muscovy (*C. moschata domestica*) (Figures 2 and 3) and mule ducks (Baéza, 2006; Pingel et al., 2012). The final live weight (LW) at slaughtering is about 2.5-3.5 kg for Pekin duck, and 3.0-4.5 kg for Muscovy duck, at 50 days and 70 days, respectively. Mule duck is a sterile hybrid between a male of Muscovy duck and a female of Pekin duck, raised mainly in Taiwan for meat production and for fatty liver (*foie gras*) production in France. For meat production, mule duck is usually slaughtered at 10 weeks of age, with a final LW about 3.8 kg (Baéza, 2006; Pingel et al., 2012).

**Figure 2.** Pekin ducks  
(*A. platyrhynchos domesticus*)  
(source: [www.backyardchickens.com](http://www.backyardchickens.com))



**Figure 3.** Muscovy duck  
(*C. moschata domestica*)  
(source: [www.poultrygaints.com](http://www.poultrygaints.com))



For what the production systems concern, ducks are mainly reared intensively in developed countries. The rearing could be in total-confinement housing or with access to pasture or water for swimming (Baéza, 2006). On the other hand, in Asia the main systems used are extensive, in integrated systems (such as fish-duck or rice-fish-duck) (Islam et al., 2004; Baéza, 2006).

## **4.1 Ducks nutritional requirements**

In order to obtain the greater body growth and their maximum feed efficiency potential, ducks need a favourable environment and nutrition. The proper amount of energy, proteins (in particular the essential amino acids), fats, minerals and vitamins, and the right balance between them, is essential for ducks' development.

### **4.1.1 Energy**

Energy is usually one of the most expensive components in feed formulation. Like any other animal, ducks require the adequate amount of energy indispensable for the maintenance of the basal metabolism, as well as for the regulation of body temperature, for their activity and growth (Adeola, 2006). In order to calculate ducks' energy requirement, in thermal neutrality conditions (25°C), the Leclercq (1990) equation can be applied as follow (Baéza, 2016):

$$E = 105 BW^{0.75} + 14 \text{ Prot} + 11 \text{ Lip}$$

Where E represents the daily energy requirement (MJ), BW is the Body Weight (kg), Prot is the amount of daily stored proteins, and Lip is the amount of daily stored lipids.

Despite there are slight differences between ducks' and chickens' energy requirements, ducks' diets are usually formulated using the energy values adopted for chickens (Baéza, 2016). The amount of metabolizable energy (ME) depends on the considered duck's breed as well as the age of the birds and the feed raw material considered. Indeed, contrarily to chickens, the apparent metabolizable energy (AME) requirements is negatively correlated with the ducks' age (Siregar et al., 1982; Baéza, 2016). On the other hand, similarly to broilers, the type of raw material considered significantly affected the apparent and true metabolizable energy values, with higher values reported for corn, sorghum, and wheat, compared to barley and triticale (Adeola, 2006; Hoai et al., 2011).

For Pekin ducks, the ME requirements recommendations are approximately 2844.65 kcal/kg and 3057.23 kcal/kg in starter and grower finisher periods, respectively (Adeola, 2006). On the other hand,

Fan et al. (2008) reported an AME requirement of 3011.85 kcal/kg on average for Pekin ducks between 2 to 6 weeks of age.

For Muscovy duck, the amount of ME should be 2794.50 kcal/kg and 2995.13 kcal/kg (INRA, 1989; Baéza, 2016). Despite ducks can regulate the amount of energy ingested by regulating their feed intake, a surplus amount of energy could affect the proportion of abdominal fat without, however, affecting the carcass composition and yields (Fan et al., 2008; Xie et al., 2010; Baéza, 2016).

#### ***4.1.2 Protein and amino acids***

As for energy, dietary protein represents one of the major costs in livestock feed formulation. The adequate amount of proteins and essential amino acids (AA) is indispensable for an efficient growth of the animals, since they are the foundation of body tissue and proteins. Adeola (2006) reported that the adequate amount of dietary crude protein (CP) for Pekin ducks is 20.5 and 17.5% in starter and grower periods, respectively. On the other hand, Muscovy duck CP requirements are slightly lower (19.0 and 16.0% of CP for starter and grower periods, respectively) (INRA, 1989). However, similarly to the energy requirements, the dietary total AA requirements for broilers are used for duck feed formulation, despite it has been reported that the AA digestibility coefficients are lower in ducks compared to broilers (Helmbrecht, 2012; Baéza, 2016).

The most limiting AA in duck feed formulation are methionine, lysine, threonine, and tryptophan (Adeola, 2006; Pingel et al., 2012; Baéza, 2016), with methionine and lysine as the first limiting AA. Moreover, it has been reported that the methionine, lysine, and tryptophan requirements are directly related to the increasing growing rate and carcass yields in Pekin duck (Pingel et al. 2012). The main protein sources currently used in poultry feeding, such as soybean and corn gluten meal, are usually poor in some AA, and particularly in methionine. Moreover, also the protein source of the essential AA could affect their availability for ducks. Adeola (2006) compared the digestibility coefficients of essential AA in corn, barley, canola meal, and soybean meal, observing significant differences between them. Indeed, the digestibility coefficients of essential AA resulted lower in barley compared to corn, and much lower in canola meal compared to soybean meal. These differences could be associated to the different AA composition and location, structure, and distribution of proteins in these raw materials (Adeola, 2006). Jeroch and Dänicke (2003) reported the AA requirements for Pekin and Muscovy ducks, indicating an amount of 0.93%, 0.49%, 0.21%, and 0.63% of lysine, methionine, tryptophane, and threonine, respectively for Pekin ducks. On the other hand, the limiting AA requirements for Muscovy ducks are 0.72%, 0.31%, 0.16%, and 0.53% of lysine, methionine, tryptophane, and threonine, respectively (Jeroch and Dänicke, 2003).

### **4.1.3 Lipids and essential fatty acids**

Oils are the most important source of energy and essential fatty acids (FA) for the birds. Lipids are extremely important for the organism, for example for the synthesis of cell membranes and hormones. The dietary lipid source and levels is an important nutritional aspect, since it can modulate meat lipid quality and FA profile (Cortinas et al., 2004; Schiavone et al., 2004; Schiavone et al., 2007). Moreover, also fat digestibility is significantly affected by their sources. Animal fat source (for example lard and tallow), particularly rich in saturated fatty acid (SFA), are less digestible compared to vegetable oils (such as soybean and palm oil), richer in unsaturated fatty acids (Danicke, 2001).

Some FA, such as linoleic, linolenic and arachidonic acids, must be provided with the diet. Indeed, these FA are considered essential, since poultry are not able to synthesize them *de novo* (Pingel et al., 2012). However, very little is known about the dietary FA requirements of ducks, and poultry in general. Only the linoleic acid requirements were determined for poultry, with 1% of linoleic acid for broiler chickens and laying hens (Ravindran, 2013).

### **4.1.4 Vitamins and minerals**

The vitamins and minerals nutritional requirement are extremely variable, depending on different factors such as the considered strain, the age of the birds and their sex, the physiological status, and the environmental conditions. Vitamins are biological substances that are useful for many biochemical reactions (Pingel et al., 2012). Despite the importance of vitamins for the maintenance of an optimal physiological status of ducks, very little is known about the correct duck's vitamins nutritional requirements. Indeed, there is a high variability in terms of recommended amounts of vitamins and some values still need to be determined exactly (Pingel et al., 2012; Baéza et al., 2016).

On the other hand, ducks' mineral requirements are better known. Minerals are essential for the skeleton constitution, for hormones synthesis and for the regulation of the osmotic pressures (Pingel et al., 2012). Calcium requirements vary from 8.5 g/kg for Pekin duck to 11-12 g/kg for Muscovy duck, while phosphorus requirements are 4 g/kg and 1.14 g/kg for Pekin and Muscovy ducks, respectively (Pingel et al., 2012).

## 5. Insects

The possibility to use insects as raw materials in feed is particularly interesting from an environmental point of view. Indeed, insects present a lower environmental impact compared to the conventional vegetable protein sources currently used in poultry feeding (such as soybean meal) in terms of land utilization, water use, and GHG emissions (van Huis and Oonincx, 2017).

In terms of land area utilization, an investigation carried out by the initiative PROteINSECT (EU) compared the amount of proteins that could be produced by 1 ha of land by insect rearing and soybean cultivation. The results highlighted that 1 ha of land could produce up to 150 tonnes of insect proteins, while the same amount land in the same time frame can produce less than 1 tonnes of soybean proteins (Dossey et al., 2016). Likewise, insects rearing could require a lower water amount compared to other farming types. Currently, few works evaluated the water requirements for insect rearing but it could be considerably low, since insects can obtain water directly from their feed (Shockley and Dossey, 2014). Moreover, some insect species are more drought-resistant than other animals (such as cattle) (van Huis et al., 2013). The insects' GHG emissions vary considerably by species, but the GHG emissions are generally lower compared to livestock (Shockley and Dossey, 2014). Oonincx et al. (2010) reported much lower insects' GHG emissions than cattle (2-122 g/kg and 2850 g/kg of mass gain for insects and cattle, respectively) and in a lower range than pigs (80-1130 g/kg of mass gain). Furthermore, also the ammonia emission levels resulted to be lower compared to conventional livestock (36-142 mg/kg and 1140-1920 mg/kg of mass gain for insects and pigs, respectively) (Oonincx et al. 2010).

One of the main advantages of insects' rearing is that they can be fed by a wide variety of organic side streams (such as fruit and vegetable wastes and plant by-products). Thus, the possibility to use organic wastes for insect rearing could be useful to manage wastes and produce, at the same time, high value edible biomass (Surendra et al., 2017). Indeed, insects are characterized by an efficient feed conversion ratio (FCR), that is associated to a poikilothermic metabolism ("cold blooded") that do not require energy for body warmth (Premalatha et al., 2011). It has been calculated that to obtain 1 kg of high-quality animal protein, 6 kg of plant proteins must be used (Pimentel and Pimentel, 2003). On the contrary, Collavo et al. (2005) calculated that insects' feed requirements are notably lower, since the production of 1 kg of live crickets (live animal weight) requires only 1.7 kg of feed. These advantages, associated to insects' rapid growth rates, short life cycles and high fecundity, make insects a promising and environmentally friendly feed ingredient for livestock (Shockley and Dossey, 2014).

With the Regulation (EU) 2017/893, European Community allowed insect-derived protein for aquaculture feeds. This Regulation partially modified the Regulation 999/2001 regarding the preventive measures against the Transmissible Spongiform Encephalopathy, in which the processed animal proteins were forbidden for livestock feed (IPIFF, 2020). Nevertheless, current Regulation 999/2001 still not authorize the use of insect-derived proteins as feed for other monogastric species, such as pigs

and poultry. Nowadays, seven insect species are authorized for aquaculture feed: black soldier fly, yellow mealworm, house fly, lesser mealworm, house cricket, banded cricket, and field cricket. Moreover, the EU legislation established the types of substrates authorized for insects' rearing, that are the same approved for other farmed animals (materials from plant origins, eggs, milk, and derived products) (IPIFF, 2020).

Legislation reforms are expected by European Commission in the future years, in order to remove the feed-ban and authorize the insect-derived proteins also for pigs and poultry feed (IPIFF, 2020).

## **5.1 Insects reared species and their nutritional value**

Many insect species are currently raised, belonging to several order. In particular, the main cultured insect species belong to Lepidoptera (butterflies and moths), Coleoptera (beetles), Diptera (mosquitoes), Heteroptera (bugs), Hymenoptera (bees and wasps), Orthoptera (grasshoppers), and others (Leppla, 2002). As previously reported, seven insect species are currently authorized in Europe for aquaculture feed, with the future possibility to be authorized also for poultry and swine in the future years. In the present paragraph an overview about these insect species and their nutritional values will be presented.

### **5.1.1 *Hermetia illucens***

The *Hermetia illucens* (HI), or black soldier fly, is a Diptera (Stratiomyidae family) originated from tropical, subtropical, and warm temperate zones of America, currently naturalized worldwide (Wang and Shelomi, 2017) (Figures 4 and 5). In optimal environmental conditions, the HI larvae hatches after four days, and one larva can process from 25 to 500 mg of substrate/daily. The HI larvae could be reared on a wide variety of organic materials, such as food and kitchen wastes, fish offal, distillers' grains, manure, and faecal sludge (Wang and Shelomi, 2017). The whole HI life cycle depends on environmental conditions, from few weeks to some months, and from quantity and quality of the substrate (Makkar et al., 2014).

As for the other insect species, the chemical composition of HI larvae depends on the rearing substrates and on the larval development stage (Barragan-Fonseca et al., 2017). The HI live larvae contain a high amount of moisture, with a dry matter (DM) content about 30% as fed. The CP and ether extract (EE) contents of HI live larvae are around 15% and 12% DM, respectively (Veldkamp and van Niekerk, 2018) (Table 2). In order to be able to manage fat and protein sources separately in feed formulations, HI manufacturers have started to produce not only the full fat HI larva meal, but also partially and highly defatted HI larva meals (Schivovone et al., 2017).

**Figure 4.** *Hermetia illucens* adult  
(source: www.entomologytoday.org)



**Figure 5.** *Hermetia illucens* larva  
(source: www.joshsfrogs.com)



The defatting process consists in the partial or total removal of the fat fraction from HI meal, by mechanical or chemical processes (Fasakin et al., 2003; Kroeckel et al., 2012). Thus, depending on the type of HI larva meal considered, the CP content can vary from 37% to 65% DM for the full fat and the highly defatted HI larva meal, respectively. On the other hand, the EE content results to be around 35% for the full fat HI meal and about 8% DM in the highly defatted HI larva meal (De Marco et al. 2015; Schiavone et al. 2017) (Table 2).

**Table 2.** Nutrient composition of HI live larvae and of a full fat, a partially and a highly defatted HI larva meals (De Marco et al. 2015; Schiavone et al. 2017; Veldkamp and van Niekerk, 2018).

| <i>Hermetia illucens</i> | DM<br>(% as fed) | CP<br>(% DM) | EE<br>(% DM) | Ash<br>(% DM) | Gross Energy<br>(MJ/ kg DM) |
|--------------------------|------------------|--------------|--------------|---------------|-----------------------------|
| Live larvae              | 32.3             | 15.6         | 12.4         | 1.6           | -                           |
| Full fat meal            | 95.7             | 36.9         | 34.3         | 13.0          | 23.8                        |
| Partially defatted meal  | 94.2             | 55.3         | 18.0         | 4.1           | 24.4                        |
| Highly defatted meal     | 98.5             | 65.5         | 4.6          | 7.8           | 21.2                        |

Note: DM: dry matter; CP: crude protein; EE: ether extract.

The AA profile of HI larvae is slightly influenced by diet, resulting always particularly rich in lysine (6-8% of CP content) (Table 3). Compared to soybean meal, the HI larvae reared on swine manure presents a similar AA profile in terms of lysine, leucine, phenylalanine, and threonine, while higher amounts of alanine, methionine, histidine, and tryptophan were observed (Barragan-Fonseca et al., 2017) (Table 3).

**Table 3.** Amino acid profile of HI larvae compared to soybean meal (% DM and g/16 g nitrogen) (Barragan-Fonseca et al., 2017).

| Amino acid    | HI larvae | Soybean meal |
|---------------|-----------|--------------|
| Alanine       | 7.7       | 4.5          |
| Arginine      | 5.6       | 7.5          |
| Aspartic acid | 11.0      | 12.1         |
| Cystine       | 0.1       | 1.2          |
| Glycine       | -         | 4.2          |
| Glutamic acid | 10.9      | 19.3         |
| Histidine     | 3.0       | 2.5          |
| Isoleucine    | 5.1       | 5.0          |
| Leucine       | 7.9       | 8.0          |
| Lysine        | 6.6       | 6.4          |
| Methionine    | 2.1       | 1.2          |
| Phenylalanine | 5.2       | 5.0          |
| Proline       | 6.6       | 5.5          |
| Serine        | 3.1       | 4.8          |
| Threonine     | 3.7       | 3.9          |
| Tryptophan    | 0.5       | -            |
| Tyrosine      | 6.9       | 3.6          |

Note: HI: *Hermetia illucens*.

The FA profile of HI larvae is mainly influenced by the FA profile of the substrate where the larvae are reared (Table 4). Generally, the amount of SFA in HI larvae is around 58-72%, while the monounsaturated fatty acid (MUFA) and polyunsaturated fatty acids (PUFA) contents represent the 19-40% of the total fat content (Barragan-Fonseca et al., 2017). Among the SFA, lauric acid (C12:0) and palmitic acid (C16:0) are the most representative. Lauric acid can vary from 26% of total FA in HI larvae reared on cattle manure, to 47% in HI larvae reared on chicken feed. Palmitic acid content results to be around 17% of total FA in HI larvae reared on cattle manure, while lower levels were observed in HI larvae reared on chicken feed (14% of total FA). The oleic acid (C18:1 n9) is the most abundant MUFA, with an amount around 26, 18, and 12% in HI larvae reared on cattle manure, cattle manure enriched with fish offal, and chicken feed respectively (Table 4) (St-Hilaire et al., 2007; Li et al., 2011; Sealey et al., 2011; Oonincx et al., 2015; Barragan-Fonseca et al., 2017).

**Table 4.** Fatty acids content (% of total FA) of HI larvae reared on different substrates (mean  $\pm$  standard deviation) (St-Hilaire et al., 2007; Li et al., 2011; Sealey et al., 2011; Oonincx et al., 2015; Barragan-Fonseca et al., 2017).

| Fatty acids              | Cattle manure  | Cattle manure +<br>fish offal | Chicken feed   |
|--------------------------|----------------|-------------------------------|----------------|
| Capric acid              | 3.1            | -                             | 0.9            |
| Lauric acid              | 26.7 $\pm$ 7.8 | 35.6 $\pm$ 2.1                | 46.8 $\pm$ 0.3 |
| Myristic acid            | 3.9 $\pm$ 1.6  | 6.4 $\pm$ 0.1                 | 7.8 $\pm$ 1.9  |
| Palmitic acid            | 16.9 $\pm$ 2.6 | 15.8 $\pm$ 2.1                | 13.8 $\pm$ 1.6 |
| Palmitoleic acid         | 5.1 $\pm$ 1.8  | 7.6                           | 3.4            |
| Stearic acid             | 5.3 $\pm$ 1.5  | 2.2 $\pm$ 0.3                 | 2.1 $\pm$ 0.1  |
| Oleic acid               | 26.1 $\pm$ 5.2 | 17.6 $\pm$ 1.6                | 12.1 $\pm$ 2.7 |
| Linoleic acid            | 4.5 $\pm$ 2.4  | 4.9 $\pm$ 1.4                 | 9.4            |
| $\alpha$ -linolenic acid | 0.2            | 0.6 $\pm$ 0.1                 | 0.7 $\pm$ 0.1  |
| Arachidonic acid         | 0.04           | 0.2                           | 0.1            |
| Eicosapentaenoic acid    | 0.07 $\pm$ 0.1 | 2.6 $\pm$ 1.2                 | -              |
| Docosapentaenoic acid    | 0.01           | 0.2 $\pm$ 0.2                 | 0.1            |

As far as the mineral content concern, the HI larvae are particularly rich in minerals and, also in this case, their mineral profile is linked to the rearing substrate. The HI larvae are particularly rich in calcium and phosphorus, with 5% DM of calcium and 1% DM of phosphorus, on average. Moreover, also the potassium content is particularly high, with 1.2% DM on average, followed by the magnesium (0.5% DM on average) (Table 5) (Arango Gutiérrez, 2005; Newton et al., 2005; Barragan-Fonseca et al., 2017).

**Table 5.** Mineral content (% DM) of HI larvae reared on different substrates (mean  $\pm$  standard deviation) (Arango Gutiérrez, 2005; Newton et al., 2005; Barragan-Fonseca et al., 2017).

| Minerals   | Poultry manure  | Swine manure | Chicken feed |
|------------|-----------------|--------------|--------------|
| Calcium    | 6.40 $\pm$ 1.98 | 5.36         | 3.14         |
| Phosphorus | 1.10 $\pm$ 0.56 | 0.88         | 1.28         |
| Magnesium  | 0.38 $\pm$ 0.01 | 0.44         | 0.79         |
| Sodium     | 0.15            | 0.13         | 0.27         |
| Potassium  | 0.65 $\pm$ 0.07 | 1.16         | 1.96         |
| Iron       | 0.07 $\pm$ 0.09 | 0.08         | 0.04         |
| Zinc       | 0.01            | 0.03         | 0.02         |
| Copper     | 0.001           | 0.003        | 0.002        |
| Manganese  | 0.04 $\pm$ 0.03 | 0.03         | 0.04         |

Finally, also the HI larvae fat-soluble vitamin profile can be modified by the rearing substrate, and vitamin E content in particular. Finke (2013) reported an amount of 9 IU/kg in HI larvae for vitamin E, while the vitamin A amount is generally less affected by the larvae substrate (< 1,000 IU/kg in HI larvae). Moreover, HI larvae are a good source of B-vitamins, with pantothenic acid, niacin, and riboflavin as the most representative (Table 6; Finke, 2013; Koutsos et al., 2019).

**Table 6.** Vitamin amount of HI larvae (Finke, 2013; Koutsos et al., 2019).

| Vitamins                        | Amount |
|---------------------------------|--------|
| Vitamin A (IU/kg)               | <1,000 |
| Vitamin D <sub>3</sub> (IU/kg)  | 100    |
| Vitamin E (IU/kg)               | 9.2    |
| Vitamin C (mg/kg)               | < 10   |
| Thiamin (mg/kg)                 | 7.7    |
| Riboflavin (mg/kg)              | 16.2   |
| Pantothenic acid (mg/kg)        | 38.5   |
| Niacin (mg/kg)                  | 71.0   |
| Pyridoxine (mg/kg)              | 6.0    |
| Folic acid (mg/kg)              | 2.7    |
| Biotin (mg/kg)                  | 0.35   |
| Vitamin B <sub>12</sub> (ug/kg) | 56     |
| Choline (mg/kg)                 | 1,100  |

### 5.1.2 *Tenebrio molitor*

The *Tenebrio molitor* (TM), known as yellow mealworm, is a Coleoptera of the Tenebrionidae family, indigenous species of Europe and currently distributed worldwide (Makkar et al., 2014) (Figures 6 and 7). The whole TM life cycle at optimal environmental conditions least from 75 to 90 days (Ribeiro et al., 2018). Since TM larvae are omnivorous, the rearing substrate can be composed by a wide variety of plant materials as well as animal products. However, the substrates are generally composed by cereal bran or meal (wheat, oats or maize) supplemented with protein sources (soybean meal, skimmed milk powder or yeast) and fresh fruits and vegetables (carrots, potatoes, lettuce) (Makkar et al., 2014; Ribeiro et al., 2018).

**Figure 6.** *Tenebrio molitor* adult  
(source: [www.permatreat.com](http://www.permatreat.com))



**Figure 7.** *Tenebrio molitor* larva  
(source: [www.reptilhouse.com](http://www.reptilhouse.com))



The CP content of TM larvae is high, with an amount of 52.2% DM (Table 7). The TM larvae AA profile is particularly good, since it is rich in lysine which content is 3.2 % DM on average (Makkar et al., 2014, Hong et al., 2020). On the contrary, methionine is less abundant, with 1% DM, on average (Hong et al., 2020). The EE content vary from 31 to 43% DM, with an amount of 30.8 % DM on average (Makkar et al., 2014, Hong et al., 2020). The fatty acid composition is mainly represented by unsaturated fatty acids (78.2% DM), while the SFA are 22.2% DM on average (Table 7). Among the SFAs, palmitic acid is the most representative (16.1-28.7% of total FAs), while oleic and linoleic acids (C18:2) are the prevalent MUFA and PUFA, respectively (27.7-43.3% and 23.1-31.0% on the total FAs for oleic and linoleic acids, respectively) (Makkar et al., 2014; Ribeiro et al., 2018). The mineral content is quite low, with the most limiting mineral represented by Ca (only 0.25% DM, Table 7) (Ribeiro et al., 2018; Hong et al., 2020).

**Table 7.** Nutrient composition and AA, FA and minerals content of TM larvae (mean  $\pm$  standard deviation) (Makkar et al., 2014; Hong et al., 2020).

| Item                      | TM larvae        |
|---------------------------|------------------|
| <i>Nutrients</i>          |                  |
| Crude protein (% DM)      | 52.25 $\pm$ 4.66 |
| Ether extract (% DM)      | 30.77 $\pm$ 5.81 |
| Ash (% DM)                | 4.43 $\pm$ 1.69  |
| Gross energy (Mj/kg DM)   | 26.8 $\pm$ 0.4   |
| <i>Amino acids</i> (% DM) |                  |
| Lysine                    | 3.18 $\pm$ 1.50  |
| Methionine                | 1.06 $\pm$ 0.55  |
| <i>Fatty acids</i> (% DM) |                  |
| Saturated fatty acids     | 22.19 $\pm$ 0.96 |
| Unsaturated fatty acids   | 78.25 $\pm$ 0.59 |
| n-6/n-3                   | 27.8 $\pm$ 36.83 |
| <i>Minerals</i> (% DM)    |                  |
| Calcium                   | 0.25 $\pm$ 0.22  |
| Phosphorus                | 0.86 $\pm$ 0.18  |

### **5.1.3 Other insect species – *Musca domestica*, *Alphitobius diaperinus*, and *Orthoptera***

In addition to the insect species mentioned above, the Regulation (EU) 2017/893 also includes *Musca domestica*, *Alphitobius diaperinus*, and three Orthoptera species suitable for aquaculture feed. *Musca domestica* (MD), known as house fly, is the most common Diptera of Muscidae family, and it is well known worldwide as pest and disease vector. In controlled conditions, the MD life cycle can last 6-10 days. The reproduction is particularly efficient, since the female can lay from 500 to 2000 eggs and the maggots do not need huge amount of substrate for their growing (it has been observed that it is possible to obtain 1500 maggots from only 450 g of fresh manure) (Hardouin and Mahoux, 2003; Makkar et al., 2014). Many rearing substrates have been tested for MD maggots growing, such as poultry and pig manure, fish and cattle guts and other animal offal and rotten fruits (Makkar et al., 2014).

The *Alphitobius diaperinus*, or lesser mealworm, is a Coleoptera (Tenebrionidae) native from the tropics, where it lives in decaying trees and bird nests. Currently, *A. diaperinus* is the most common beetle that can be found in poultry and pig litter and manure (Salin et al., 2000). Moreover, it is a flour and cereal pest, feeding on wheat, barley, rice, oat, soybean, pea, and peanuts (Dinev, 2013). The female can lay

over 2,000 eggs and, after 4-7 days the larvae hatches. Depending on environmental conditions, after 40-100 days the larvae develop into adults (Dinev, 2013).

Finally, house cricket (*Acheta domesticus*), banded cricket (*Grylloides sigillatus*), and field cricket (*Gryllus assimilis*) are the three Orthoptera species approved for aquaculture feed. Crickets are omnivorous and they can be reared on a wide variety of organic materials. Moreover, crickets are good bio converters, requiring only 2 kg of feed for 1 kg of body weight (BW; van Huis et al., 2013; Makkar et al., 2014). The CP content of the above-mentioned species is quite high. The MD larvae contain 55% DM of CP on average, while *A. diaperinus* larvae present a CP level around 61.5% DM (Pieterse and Pretorius, 2014; Rumbos et al., 2019). On the other hand, also Orthoptera species are rich in CP, with values between 50 to 65% DM (Makkar et al., 2014).

The EE content of MD larvae is around 21.9% DM, varying from 14 to 27% DM, with a FA profile rich in palmitoleic, oleic, and linoleic acid (Pieterse and Pretorius, 2014). Moreover, it has been reported to linoleic and linolenic acids (Hwangbo et al., 2009). Regarding *A. diaperinus*, the EE content is 21% DM on average, and it is particularly rich in linoleic acid (16.8–36.4 % of total FA) (Rumbos et al., 2019). As for the other insect species, the Orthoptera EE content is extremely variable, ranging from less than 5% DM to more than 20% DM, and the FA profile depends on the considered species (Makkar et al., 2014). For example, house crickets are rich in linoleic, palmitic, and oleic acids (38.0, 23.4, and 23.8% of total FA, respectively; Makkar et al., 2014).

# **Chapter 2**

## ***Experimental trials***

## **6. Aim of the PhD project**

The aims of the present PhD projects are the evaluation of the dietary inclusion of HI meal and live HI and TM larvae in Muscovy ducks.

Dietary HI larva meal was included in Muscovy duck diet in order to investigate the nutritional effects of this new feed ingredient, while HI and TM live larvae were provided in order to investigate their effects as environmental enrichment.

Different partnerships [University of Turin (Italy); National Research Council (Italy); University of Padua (Italy); University of Genoa (Italy); Veterinary Medical Research Institute for Piemonte, Liguria and Valle d'Aosta (Italy); University of Murcia (Spain); Slovak Academy of Sciences (Slovak Republic)] contributed to the present PhD research in order to evaluate the growth and slaughtering performance, digestibility, welfare and meat quality and safety, and gut health assessment of insects fed Muscovy ducks.

Growth and slaughtering performance, digestibility, behavioural observations and the most part of meat quality parameters were directly evaluated by the PhD student.

## **7. Materials and Methods**

A total of two experimental trials were performed in the present PhD project. The first experimental trial was performed during the first year, while the second trial was conducted in the second year.

The experimental protocol for the two experiment was outlined according to the guidelines of the current European and Italian laws on the care and use of experimental animals (European Directive 86/609/EEC), and it was approved by the Bioethical Committee of the University of Turin (Italy; prot. no. 380576) on the 4<sup>th</sup> of December 2017.

The two experimental trials will be defined as Exp 1 for the first experiment (increasing levels of HI larvae meal), and Exp 2 for the second experiment (dietary inclusion of HI and TM live larvae).

### **7.1 Birds and husbandry**

Both the experimental trials were carried out at the poultry facility of the Department of Agricultural, Forest and Food Science of the University of Turin (Italy). The poultry house used for both the experimental trials was 7 m wide × 50 m long × 7 m high, with an automatic ventilation system and equipped with a waterproof floor and walls. Each pen was 1.20 m wide × 2.20 m long and rice hulls was used as bedding. The birds were heated by infrared lamps during the first three weeks of life. Until day 3 of the trial (6 days old of birds) the daily lighting schedule was 23L:1D and thereafter the dark period was progressively increased to 6 h and maintained constant until slaughtering. Moreover, the environmental parameters were monitored daily during the whole period of the trial.

#### ***7.1.1 Exp 1***

For the Exp 1, a total of 192 females Muscovy ducklings (Canedins R71 L White, Grimaud Freres Selection, France) at 3 days old were distributed in 24 pens and randomly assigned to 4 dietary treatments. Each experimental treatment consisted in 6 pens as replicates with 8 birds per pen. The birds were distributed in order to have an average LW of  $71.32 \pm 2.95$  g.

#### ***7.1.2 Exp 2***

For the Exp 2, 126 females Muscovy ducklings (Canedins R61 Barred blue, Grimaud Freres Selection, France) were used. The birds arrived at 3 days old and were allotted in 18 pens (6 replicate/treatment, 7 birds/pen), with an average LW of  $79.8 \pm 3.3$  g. The birds were then assigned to 3 experimental treatments.

## **7.2 Growth Performance**

At their arrival, the birds were individually labelled with a wing mark. Throughout the trial, mortality and clinical signs of illness were monitored daily.

### ***7.2.1 Exp 1***

The LW of the animals was recorded at an individual level at the beginning (average LW of  $71.32 \pm 2.95$  g) of the trial and at the end of each feeding phase (3, 17, 38 and 50 days of age). The average daily gain (ADG) and daily feed intake (DFI) were recorded at the end of each growth period at a pen level, and the FCR was then calculated.

At the end of the trial ADG, DFI and FCR were calculated for the overall experimental period at a pen level. All the measurements were made using electronic scales (Sartorius – Signum®, Bovenden, Germany).

### ***7.2.2 Exp 2***

The birds were weighed at the beginning of the trial (average LW of  $79.8 \pm 3.3$  g), at the end of the started period (31 days of age), and at the end of the trial (55 days of age). The ADG, DFI, and FCR (only feed, without larvae provided) were calculated at the end of each growth period and for the overall experimental trial, at a pen level. All the measurements were made using electronic scales (Sartorius – Signum®, Bovenden, Germany).

## 7.3 Diets

### 7.3.1 Exp 1

In order to evaluate the effects of HI meal inclusion in the diets, HI meal was included in substitution of corn gluten meal, that has a nutritional value comparable to HI meal. In particular, four dietary treatments were formulated in which increasing levels of a partially defatted HI larva meal (Hermetia Deutschland GmbH & Co KG, Baruth/Mark, Germany) were included. The control group was fed a diet without insect meal (HI0; 9% inclusion of corn gluten meal), while in the other three diets the 3%, 6% and 9% of HI larva meal was included (HI3, HI6, and HI9 diets, respectively) (Table 8).

Moreover, in order to meet the ducks' nutritional requirements, a 3-phase feeding program was applied: starter diet (from 3 to 17 days old), grower diet (from 18 to 38 days old) and finisher diet (from 39 to 51 days old). During the trial feed and water were provided *ad libitum*.

The diets were isonitrogenous and isoenergetic and were formulated using the apparent metabolizable energy nitrogen corrected (AMEn) values calculated by Schiavone et al. (2017) for a partially defatted HI larva meal, and according to INRA (2004) for the other ingredients, both for broiler chickens.

**Table 8.** Ingredients (g/kg as fed) of the experimental diets of the Exp 1, with increasing levels of *Hermetia illucens* (HI) meal.

| Ingredients                         | Starter period (days 3 to 17) |             |             |             | Grower period (days 18 to 38) |             |             |             | Finisher period (days 39 to 50) |             |             |             |
|-------------------------------------|-------------------------------|-------------|-------------|-------------|-------------------------------|-------------|-------------|-------------|---------------------------------|-------------|-------------|-------------|
|                                     | HI0                           | HI3         | HI6         | HI9         | HI0                           | HI3         | HI6         | HI9         | HI0                             | HI3         | HI6         | HI9         |
| Corn meal                           | 600.0                         | 600.0       | 600.0       | 600.0       | 638.0                         | 638.0       | 638.0       | 638.0       | 670.0                           | 670.0       | 670.0       | 670.0       |
| Soybean meal                        | 212.0                         | 212.0       | 212.0       | 212.0       | 160.0                         | 160.0       | 160.0       | 160.0       | 100.0                           | 100.0       | 100.0       | 100.0       |
| HI larva meal                       | 0.0                           | 30.0        | 60.0        | 90.0        | 0.0                           | 30.0        | 60.0        | 90.0        | 0.0                             | 30.0        | 60.0        | 90.0        |
| Bran                                | 42.5                          | 42.5        | 42.5        | 42.5        | 36.3                          | 36.3        | 36.3        | 36.3        | 66.2                            | 66.2        | 66.2        | 66.2        |
| Corn gluten meal                    | 90.0                          | 60.0        | 30.0        | 0.0         | 90.0                          | 60.0        | 30.0        | 0.0         | 90.0                            | 60.0        | 30.0        | 0.0         |
| Soybean oil                         | 16.5                          | 16.5        | 16.5        | 16.5        | 28.5                          | 28.5        | 28.5        | 28.5        | 34.5                            | 34.5        | 34.5        | 34.5        |
| Dicalcium phosphate                 | 10.0                          | 10.0        | 10.0        | 10.0        | 13.0                          | 13.0        | 13.0        | 13.0        | 4.0                             | 4.0         | 4.0         | 4.0         |
| Calcium carbonate                   | 8.0                           | 8.0         | 8.0         | 8.0         | 14.0                          | 14.0        | 14.0        | 14.0        | 17.4                            | 17.4        | 17.4        | 17.4        |
| Sodium chloride                     | 2.5                           | 2.5         | 2.5         | 2.5         | 2.5                           | 2.5         | 2.5         | 2.5         | 2.5                             | 2.5         | 2.5         | 2.5         |
| Sodium bicarbonate                  | 2.0                           | 2.0         | 2.0         | 2.0         | 2.0                           | 2.0         | 2.0         | 2.0         | 2.0                             | 2.0         | 2.0         | 2.0         |
| DL-methionine                       | 2.5                           | 2.5         | 2.6         | 2.8         | 1.7                           | 1.8         | 1.9         | 2.2         | 0.3                             | 0.4         | 0.5         | 0.8         |
| L-lysine HCl                        | 3.9                           | 3.9         | 3.8         | 3.6         | 3.9                           | 3.8         | 3.7         | 3.4         | 3.0                             | 2.9         | 2.8         | 2.5         |
| Mineral-vitamin premix <sup>2</sup> | 5.0                           | 5.0         | 5.0         | 5.0         | 5.0                           | 5.0         | 5.0         | 5.0         | 5.0                             | 5.0         | 5.0         | 5.0         |
| Choline chloride                    | 0.1                           | 0.1         | 0.1         | 0.1         | 0.1                           | 0.1         | 0.1         | 0.1         | 0.1                             | 0.1         | 0.1         | 0.1         |
| Optifos 250 bro <sup>3</sup>        | 1.0                           | 1.0         | 1.0         | 1.0         | 1.0                           | 1.0         | 1.0         | 1.0         | 1.0                             | 1.0         | 1.0         | 1.0         |
| Avizyme 1500 x <sup>4</sup>         | 1.0                           | 1.0         | 1.0         | 1.0         | 1.0                           | 1.0         | 1.0         | 1.0         | 1.0                             | 1.0         | 1.0         | 1.0         |
| Titanium dioxide                    | 3.0                           | 3.0         | 3.0         | 3.0         | 3.0                           | 3.0         | 3.0         | 3.0         | 3.0                             | 3.0         | 3.0         | 3.0         |
| <b>Total</b>                        | <b>1000</b>                   | <b>1000</b> | <b>1000</b> | <b>1000</b> | <b>1000</b>                   | <b>1000</b> | <b>1000</b> | <b>1000</b> | <b>1000</b>                     | <b>1000</b> | <b>1000</b> | <b>1000</b> |

<sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*.

<sup>2</sup>Mineral-vitamin premix per kg: vitamin A (retinyl acetate), 12,500 IU; vitamin D3 (cholecalciferol), 3,500 IU; vitamin E (DL- $\alpha$ -tocopheryl acetate), 40 mg; vitamin K (menadione sodium bisulfite), 2.0 mg biotin, 0.20 mg; thiamine, 2.0 mg; riboflavin, 6.0 mg; pantothenate, 15.21 mg; niacin, 40.0 mg; choline, 750.0 mg pyridoxine, 4.0 mg; folic acid, 0.75 mg; vitamin B12, 0.03 mg; Mn, 70 mg; Zn, 62.15 mg; Fe, 50.0 mg; Cu, 7.0 mg; I, 0.25 mg; Se, 0.25 mg.

<sup>3</sup>Optifos 250 bro: Phytase (EC 3.1.3.26) (250 OTU/kg diet), Huvepharma, Sofia, Bulgaria.

<sup>4</sup>Avizyme 1505X: Complex of Endo 1-4-Beta- Xylanase (EC 3.2.1.8) (256 U/kg), Subtilisin (Ec 3.4.21.62) (2560 U/kg diet) and Alpha-Amylase (EC3.2.1.1) (1472 U/kg diet), Danisco Animal Nutrition, Marlborough, Wiltshire, UK

### ***7.3.2 Exp 2***

In the Exp 2 live insects' larvae were provided at the 5% of the expected average DFI (on fresh matter basis, calculated on the basis of the results obtained in the first experiment). A commercial based diet was used for all the experimental treatments, provided by Borello Mangimi s.r.l. (Bra, CN, Italy). A 2-feeding phase program was applied: started diet (from 3 to 31 days old), and grower-finisher period (from 32 to 55 days old). Three experimental treatments were designed: control group (C, fed with commercial feed); HI group (fed with commercial feed + 5% of live HI larvae); TM group (fed with commercial feed + 5% of live TM larvae). The composition of the experimental diets and their calculated nutrient compositions are reported in Table 9. The live insects' larvae were provided daily at the same time (10.00 am) in a plate. In order to evaluate the behavioural effects due to the larvae consumption, the control group received an empty plate at the same time, thus to have the same interactions with the operator in all the groups. The larvae consumption times were recorded daily from the 4<sup>th</sup> day of trial (6 days old) until the end of the experiment, for every pen by stopwatch.

**Table 9.** Ingredients (g/kg as fed) and nutrient composition (calculated) of the experimental diets of the Exp 2.

| Ingredients                         | Starter period<br>(days 3 to 31) | Grower-finisher period<br>(days 32 to 55) |
|-------------------------------------|----------------------------------|-------------------------------------------|
| Corn meal                           | 417.9                            | 541.3                                     |
| Soybean meal                        | 292.4                            | 233.7                                     |
| Bran                                | 53.4                             | 60.0                                      |
| Common wheat                        | 150.0                            | 57.8                                      |
| Wheat meal                          | 34.4                             | 50.0                                      |
| Soybean oil                         | 10.0                             | 12.0                                      |
| Calcium carbonate                   | 15.7                             | 22.9                                      |
| Dicalcium phosphate                 | 12.3                             | 9.9                                       |
| Sodium bicarbonate                  | 2.5                              | 2.1                                       |
| Sodium chloride                     | 2.0                              | 1.9                                       |
| DL-methionine                       | 2.5                              | 1.8                                       |
| L-lysine HCl                        | 0.9                              | 1.7                                       |
| Mineral-vitamin premix <sup>1</sup> | 4.0                              | 3                                         |
| Optifos 250 bro <sup>2</sup>        | 1.0                              | 1                                         |
| Avizyme 1500 x <sup>3</sup>         | 1.0                              | 1                                         |
| <b>Total</b>                        | <b>1000</b>                      | <b>1000</b>                               |
| <i>Nutrient composition</i>         |                                  |                                           |
| ME (kcal/kg)                        | 2696.1                           | 2741.9                                    |
| CP (%)                              | 19.5                             | 17.2                                      |
| EE (%)                              | 3.6                              | 4.1                                       |
| Ca (%)                              | 1.1                              | 1.2                                       |
| P (%)                               | 1.1                              | 0.9                                       |

Note: ME: metabolizable energy; CP: crude protein; EE: Ether extract.

<sup>1</sup>Mineral-vitamin premix per kg: vitamin A (retinyl acetate), 12,500 IU; vitamin D3 (cholecalciferol), 3,500 IU; vitamin E (DL- $\alpha$ -tocopheryl acetate), 40 mg; vitamin K (menadione sodium bisulfite), 2.0 mg biotin, 0.20 mg; thiamine, 2.0 mg; riboflavin, 6.0 mg; pantothenate, 15.21 mg; niacin, 40.0 mg; choline, 750.0 mg pyridoxine, 4.0 mg; folic acid, 0.75 mg; vitamin B12, 0.03 mg; Mn, 70 mg; Zn, 62.15 mg; Fe, 50.0 mg; Cu, 7.0 mg; I, 0.25 mg; Se, 0.25 mg.

<sup>2</sup>Optifos 250 bro: Phytase (EC 3.1.3.26) (250 OTU/kg diet), Huvepharma, Sofia, Bulgaria.

<sup>3</sup>Avizyme 1505X: Complex of Endo 1-4-Beta- Xylanase (EC 3.2.1.8) (256 U/kg), Subtilisin (Ec 3.4.21.62) (2560 U/kg diet) and Alpha-Amylase (EC3.2.1.1) (1472 U/kg diet), Danisco Animal Nutrition, Marlborough, Wiltshire, UK.

## 7.4 Chemical analysis of the HI meal and experimental diets

### 7.4.1 Exp 1

In order to perform the chemical analysis, the experimental diets were ground to a diameter of 0.5 mm and stored in airtight plastic bags. The HI larva meal and the experimental diets were analysed for DM (AOAC, method number #934.01), ash (AOAC, method number #942.05), CP (AOAC, method number #984.13), neutral detergent fiber (NDF; AOAC, method number #2002.04), and acid detergent fiber (ADF; AOAC method number #973.18) (AOAC, 2002). The EE (AOAC, method number #2003.05) was determined according to International AOAC (2003). The chitin content of HI larva meal was determined according to Finke (2007) using ADF adjusted for its nitrogen content (chitin content of HI larva meal: 6.43%). The results of the chemical analysis of HI larva meal and the experimental diets are reported in Table 10.

The lipid extraction and FAs profiling of the HI larva meal and the experimental diets were carried out at the laboratory of the Department of Animal Medicine, Production and Health, University of Padua, Legnaro (Italy) according to the method of Christie (1982). The total fat was extracted from fresh aliquots using an accelerated solvent extraction (ASE®, Dionex, Sunnyvale, CA, USA, Application Note 334), using two extraction cycles with petroleum ether as a solvent at 125°C and 10.3 Mpa, a 6-min heating phase and a 2-min extraction phase. Then, 10 ml of NaSO<sub>4</sub> (0.47% in H<sub>2</sub>O) was added to the extracted lipids. The samples were kept at 4°C for 30 min and the supernatant (constituted by petroleum ether and lipids) was collected in another previously weighed vial. Dry evaporation in an N<sub>2</sub> stream (Genevac EZ-2, SP Industries, Warminster, PA, USA) was applied. The residual samples (extracted lipids in vials) were weighed before adding 2 ml H<sub>2</sub>SO<sub>4</sub> 2% in methanol (Christie, 1982). The vials were stored overnight at 50°C in a heater. Then, the lipid rate was calculated, and hexane (1 ml hexane/20 mg lipids) and potassium bicarbonate 2% (5 ml) were added. The thus obtained samples were centrifuged, stored at 4°C for 30 min, and the supernatant was sampled for analysis using an Agilent 7890A GC System (Agilent Technologies, Santa Clara, CA, USA), with an Autosampler 7693 (injection 1 µl); a flow modulator Agilent G3486A CFT for GC × GC (modulation period 3.00 sec, sample time 2.870 sec); a detector F.I.D. heater at 250°C. Supelco SP-2560 (Sigma-Aldrich, St. Louis, MO, USA) (75 m × 180 µm internal diameter, 0.14 µm film thickness, flow 0.25 ml/min) and Agilent J&W HP5ms (3.8 m × 250 µm internal diameter, 0.25 µm film thickness) were used with hydrogen as the carrier (split inlet, heater at 270°C, mode Pulse Split 25 psi until 0.30 min, split ratio 160:1, 40 ml/min). The temperature of the oven was set at 40°C, held for 2 min, raised to 170°C at a rate of 50°C/min, and held for 25 min, raised to 250°C at a rate of 2°C/min, and held for 14 min. The GC IMAGE software, Version 2.2b0 GC×GC (Zoex Corporation, Houston TX, USA) was used for data elaboration. The FAs were identified by comparing the retention time of a standard 52 FA methyl ester (FAME) mixture (GLC 463, NU-CHEK

PREP Elysian, MN, USA,). Individual FAMEs were expressed as the percentage of the total area of eluted FAMEs (Table 10).

The mineral analyses of the HI larva meal and experimental diets were performed at the Institute of Animal Physiology CBs, Slovak Academy of Sciences, the Slovak Republic. The Zn, Fe, Mn and Cu contents in the feed were determined using a double-beam atomic absorption spectrophotometer (AA-7000 Series, Shimadzu Co., Kyoto, Japan), using the certificate reference materials of poultry feed LGC-7173 (LGC Ltd., Teddington, UK) to verify instrument accuracy (Gresakova et al., 2016). The mineral profile of the HI larva meal was determined by using inductively coupled plasma mass spectrometry or optical emission spectrometry (ICP-MS, Agilent 7900; ICP-OES, Agilent 5100, Agilent Technologies, US) (Gresakova et al., 2016). The mineral concentrations in all the samples were expressed as mg/kg (Table 10).

The HI larva meal was analysed in order to determine the concentration of the trace elements (Co, Cr, Ni, Se), As and the heavy metal (Hg, Cd, Pb) using the MI 351 Rev. 1/2015 accredited test (UNI EN 13804:2002, UNI EN 15763:2010 and UNI EN 13805:2014), as described by Schiavone et al. (2019). Aliquots of the samples (0.5 g) were weighed in allotted digestion vessels and a mixture of deionized water, nitric acid and hydrogen peroxide was added. The vessels were capped, and the contents digested under high temperature and pressure using a single reaction chamber microwave digester system. The resulting solutions were transferred to pre-marked, acid-cleaned, plastic test tubes and diluted with deionized water. The metal content was determined by means of the ICP-MS technique (inductively coupled plasma spectrometry). Multi-element measurements were made using an Agilent 7700x ICP-MS (Agilent Technologies) with a collision cell. The results of the trace elements, As and the heavy metal concentrations in HI larva meal are reported in Table 11, expressed as mg/kg of sample or mg/kg of sample with 12% humidity.

**Table 10.** Chemical, fatty acid and mineral compositions of the experimental diets<sup>1</sup> containing partially defatted HI larvae meal.

| Items                                           | HI meal | Starter period (days 3 to 17) |       |       |       | Grower period (days 18 to 38) |       |       |       | Finisher period (days 39 to 50) |       |       |       |
|-------------------------------------------------|---------|-------------------------------|-------|-------|-------|-------------------------------|-------|-------|-------|---------------------------------|-------|-------|-------|
|                                                 |         | HI0                           | HI3   | HI6   | HI9   | HI0                           | HI3   | HI6   | HI9   | HI0                             | HI3   | HI6   | HI9   |
| <i>Chemical composition</i>                     |         |                               |       |       |       |                               |       |       |       |                                 |       |       |       |
| DM (g/kg)                                       | 92.4    | 89.3                          | 89.8  | 90.1  | 89.7  | 88.8                          | 89.2  | 89.1  | 89.1  | 88.7                            | 89.0  | 89.3  | 88.9  |
| CP (% DM)                                       | 56.7    | 25.1                          | 24.7  | 25.2  | 24.8  | 23.0                          | 22.5  | 22.5  | 22.4  | 20.2                            | 20.2  | 20.1  | 20.1  |
| EE (% DM)                                       | 10.7    | 4.7                           | 4.8   | 4.9   | 5.1   | 6.2                           | 6.2   | 6.4   | 6.6   | 7.3                             | 7.4   | 7.6   | 7.7   |
| NDF (% DM)                                      | 16.3    | 12.7                          | 12.3  | 12.7  | 12.4  | 12.9                          | 13.1  | 12.6  | 12.7  | 12.7                            | 13.1  | 12.7  | 12.9  |
| ADF (% DM)                                      | 10.0    | 3.4                           | 3.5   | 3.7   | 3.3   | 3.5                           | 3.5   | 3.7   | 3.5   | 3.4                             | 3.5   | 3.5   | 3.4   |
| Ash (% DM)                                      | 16.4    | 5.6                           | 6.0   | 5.6   | 5.8   | 7.8                           | 7.5   | 7.5   | 8.0   | 6.5                             | 6.4   | 6.9   | 6.7   |
| AMEn (kcal/kg)                                  |         | 2897                          | 2892  | 2888  | 2884  | 2994                          | 2990  | 2986  | 2981  | 3052                            | 3048  | 3044  | 3040  |
| <i>Fatty acid composition (% of total FAME)</i> |         |                               |       |       |       |                               |       |       |       |                                 |       |       |       |
| C12:0                                           | 49.70   | 0.07                          | 2.73  | 5.49  | 8.11  | 0.07                          | 2.57  | 4.82  | 7.67  | 0.07                            | 2.07  | 4.82  | 6.35  |
| C14:0                                           | 10.10   | 0.16                          | 0.60  | 1.13  | 1.65  | 0.14                          | 0.57  | 0.96  | 1.46  | 0.10                            | 0.48  | 0.99  | 1.26  |
| C16:0                                           | 13.29   | 14.99                         | 13.91 | 13.71 | 13.62 | 13.99                         | 13.69 | 13.36 | 13.06 | 13.78                           | 13.53 | 13.01 | 12.71 |
| C18:0                                           | 2.04    | 2.83                          | 2.62  | 2.65  | 2.53  | 2.75                          | 2.64  | 2.58  | 2.52  | 2.67                            | 2.63  | 2.71  | 2.70  |
| C16:1 n-7                                       | 3.09    | 0.15                          | 0.29  | 0.44  | 0.57  | 0.15                          | 0.29  | 0.39  | 0.53  | 0.14                            | 0.26  | 0.42  | 0.50  |
| C18:1 n-9                                       | 9.28    | 25.77                         | 24.20 | 23.50 | 22.42 | 23.93                         | 22.94 | 22.19 | 21.29 | 23.10                           | 22.85 | 21.74 | 21.94 |
| C18:1 n-7                                       | 0.44    | 1.10                          | 1.03  | 1.00  | 1.03  | 1.21                          | 1.15  | 1.12  | 1.08  | 1.22                            | 1.16  | 1.10  | 1.07  |
| C18:2 n-6                                       | 6.93    | 49.08                         | 48.80 | 46.05 | 44.27 | 51.46                         | 49.84 | 48.07 | 46.06 | 52.25                           | 50.36 | 47.82 | 46.28 |
| C18:3 n-3                                       | 0.86    | 3.27                          | 3.40  | 3.35  | 3.29  | 4.02                          | 4.13  | 4.05  | 4.06  | 4.48                            | 4.43  | 5.28  | 4.98  |
| ΣSFA <sup>2</sup>                               | 76.53   | 19.00                         | 20.72 | 23.90 | 26.88 | 17.70                         | 20.22 | 22.52 | 25.56 | 17.31                           | 19.44 | 22.25 | 23.75 |
| ΣMUFA <sup>2</sup>                              | 13.21   | 27.50                         | 25.99 | 25.42 | 24.48 | 25.76                         | 24.83 | 24.14 | 23.34 | 24.88                           | 24.73 | 23.65 | 23.73 |
| ΣPUFA <sup>2</sup>                              | 7.90    | 52.55                         | 52.45 | 49.64 | 47.78 | 55.77                         | 54.20 | 52.40 | 50.26 | 56.98                           | 55.02 | 53.35 | 51.52 |
| Σn-3 <sup>2</sup>                               | 0.89    | 3.31                          | 3.44  | 3.38  | 3.32  | 4.08                          | 4.16  | 4.091 | 4.10  | 4.52                            | 4.46  | 5.30  | 5.00  |
| Σn-6 <sup>2</sup>                               | 6.96    | 49.12                         | 48.89 | 46.14 | 44.34 | 51.56                         | 49.93 | 48.18 | 46.10 | 52.34                           | 50.43 | 47.92 | 46.38 |
| Σn-6/Σn-3                                       | 7.85    | 14.83                         | 14.22 | 13.65 | 13.35 | 12.65                         | 12.00 | 11.78 | 11.25 | 11.58                           | 11.32 | 9.04  | 9.27  |
| ΣPUFA/ΣSFA                                      | 0.10    | 2.77                          | 2.53  | 2.08  | 1.78  | 3.15                          | 2.68  | 2.33  | 1.97  | 3.29                            | 2.83  | 2.17  | 2.17  |

Table 10. *Continue*

| Items                                       | HI meal | Starter period (days 3 to 17) |       |       |       | Grower period (days 18 to 38) |       |       |       | Finisher period (days 39 to 50) |       |       |        |
|---------------------------------------------|---------|-------------------------------|-------|-------|-------|-------------------------------|-------|-------|-------|---------------------------------|-------|-------|--------|
|                                             |         | HI0                           | HI3   | HI6   | HI9   | HI0                           | HI3   | HI6   | HI9   | HI0                             | HI3   | HI6   | HI9    |
| <i>Analysed mineral composition (mg/kg)</i> |         |                               |       |       |       |                               |       |       |       |                                 |       |       |        |
| Zn                                          | 172.7   | 116.5                         | 124.0 | 132.6 | 120.6 | 103.9                         | 113.7 | 113.9 | 125.6 | 122.1                           | 101.5 | 133.3 | 146.2  |
| Fe                                          | 218.7   | 191.4                         | 185.4 | 193.2 | 180.5 | 154.6                         | 160.3 | 169.4 | 186.0 | 146.6                           | 113.5 | 140.1 | 147.2  |
| Mn                                          | 255.2   | 100.4                         | 119.5 | 123.5 | 115.7 | 113.6                         | 116.6 | 110.6 | 153.4 | 114.0                           | 96.1  | 137.6 | 146.35 |
| Cu                                          | 14.9    | 15.6                          | 18.0  | 18.0  | 14.9  | 13.9                          | 17.4  | 14.7  | 17.0  | 14.7                            | 11.0  | 16.6  | 14.9   |

Note: HI: *Hermetia illucens*; DM: dry matter; CP: crude protein; EE: Ether extract; NDF: neutral detergent fiber; ADF: acid detergent fiber; AMEn: apparent metabolisable energy nitrogen corrected; FAME: fatty acid methyl esters; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

<sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*. <sup>2</sup>Including minor FA.

**Table 11.** Trace elements, As and heavy metal concentrations in the HI larva meal.

| Items            | HI meal | MRL<br>(Directive 2002/32/EC) |
|------------------|---------|-------------------------------|
| Co (mg/kg)       | 0.13    | Not legislated                |
| Cr (mg/kg)       | 0.57    | Not legislated                |
| Ni (mg/kg)       | 0.73    | Not legislated                |
| Se (mg/kg)       | 0.37    | Not legislated                |
| As (mg/kg 12% h) | 0.08    | 2                             |
| Cd (mg/kg 12% h) | 0.40    | 2                             |
| Hg (mg/kg 12% h) | 0.02    | 0.1                           |
| Pb (mg/kg 12% h) | 0.15    | 10                            |

Note: HI: *Hermetia illucens*; MRL: Maximum Residue Limit; mg/kg 12% h: mg/kg feed with 12% humidity.

## 7.5 Digestibility trial

### 7.5.1 Exp 1

The digestibility trial was performed at the end of each feeding phase using titanium dioxide (TiO<sub>2</sub>, 3 g/kg) as an indigestible marker in each experimental diet (Table 8), in order to evaluate the apparent total tract digestibility coefficients (ATTDC). The Kaczmarek et al. (2005) method was used to collect the excreta, with slight modifications, as reported by Dabbou et al. (2018a). Briefly, all the birds were removed from each pen and housed in wire-mesh cages (n=6 replicates) for approximately 1 h per day for four consecutive days to collect fresh excreta samples.

After collection, the excreta samples, cleaned from feathers and litter, were immediately frozen at -20°C. At the end of each collection period, the excreta were pooled, lyophilized, grounded and stored at 4°C. All the analyses were carried out on two replicates for each sample. The ATTDC was evaluated for DM, CP, EE and organic matter (OM). The uric acid (UA) content in the excreta samples was determined spectrophotometrically (UNICAN UV-Vis Spectrometry, Helios Gamma, the United Kingdom) according to the Marquardt (1983) method. As the nitrogen contained in uric acid is the 33.33%, the CP amount in the excreta (*CP corrected*) was calculated using the excreta CP, corrected for UA as follows:

$$CP \text{ corrected} = (\text{total nitrogen} - \text{UA-nitrogen}) \times 6.25$$

The TiO<sub>2</sub> content was measured on a UV spectrophotometer (UNICAN UV-vis Spectrometry, Helios Gamma, the United Kingdom) following the Myers et al. (2004) method.

Finally, the ATTDC of the dietary nutrients was calculated using the method of Nalle et al. (2012) as follow:

$$ATTDC X_{diet} = \left[ \frac{(\text{Total } X \text{ ingested} - \text{total } X \text{ excreted})}{\text{total } X \text{ ingested}} \right]$$

$$Digestibility = \left[ \frac{(\% X_{diet} / \% TiO_{diet}) - (\% X_{excreta} / \% TiO_{excreta})}{(\% X_{diet} / \% TiO_{diet})} \right]$$

where X represents DM, CP, EE or OM.

## 7.6 Behavioural observations

### 7.6.1 Exp 2

The behavioural observations of the Exp 2 were performed by video recordings. Video recordings were taken on 3 replicate/treatment every week during the trial. The video recordings were made in 3 periods during the day as follow: the hour before insects' larvae administration (T1, from 9.00 to 10.00 am), the hour during the larvae provision (T2, from 10.00 to 11.00 am), and the hour after insects' larvae administration (T3, from 11.00 to 12.00 am). For every hour, the behaviour of all the ducks was observed, according to the ethogram in Table 12. The behavioural observations were made using BORIS software (version 7.9.7; Friard and Gamba, 2016). The observed behaviour events were divided between state and point events. State events were all the behaviours that had a duration in time, while point events were all the behaviours that had no duration (Table 12). State events were then reported as a mean expressed on the total hour of observation, while the point events were expressed as a frequency (%) on the total hour of observation.

**Table 12.** Ethogram of foraging, activity, feather caring and aggressivity behaviours.

| Behaviour category | Behaviour       | Event | Description                                 |
|--------------------|-----------------|-------|---------------------------------------------|
| Foraging           | Eat             | State | Pecking the feeder (other than insect tray) |
|                    | Eat insects     | State | Pecking the insects                         |
|                    | Drink           | State | Pecking the bell drinker                    |
|                    | Peck object     | State | Pecking objects other than feed or water    |
| Activity           | Walk            | State | Walking, running, or trotting               |
|                    | Stand           | State | Standing without activity                   |
|                    | Sleep           | State | Laying down sleeping or without activity    |
| Feather caring     | Smooth feathers | State | Arranging feathers with the beak            |
|                    | Shake           | Point | Rapid shaking of the head, body or tail     |
|                    | Stretch         | Point | Stretching movements of neck, wings or legs |
|                    | Flap wings      | Point | Beating the air with the wings              |
| Aggressivity       | Attack          | Point | Pecking a conspecific                       |

## **7.7 Blood analysis**

The blood samples were collected from the jugular vein of the slaughtered birds [12 ducks per treatment (two birds per pen) for both Exp 1 and Exp 2]. For both the experimental trials, an aliquot of 2.5 mL was placed in an EDTA tubes and 2.5 mL in a serum-separating tube. A blood smear was prepared from a droplet without any anticoagulant. The total red (erythrocytes) and white (leukocytes) cell counts were determined in an improved Neubauer haemocytometer after mixing with a Natt-Herrick solution in a 1 to 200 ratio, as reported by Natt and Herrick (1952). The blood smears were stained with May-Grünwald and Giemsa–Romanowski stains. One hundred white blood cells were evaluated per smear to determine the heterophils to lymphocytes ratio (H/L), while the number of blood cell types was determined according to Campbell (1995).

The serum-separating tubes were left in a standing position at room temperature for approximately two hours, until the formation of a blood clot. Then, the tubes were centrifugated at 700 x g for 15 min and the obtained serum was immediately frozen at -80°C. The total protein was quantified using the “biuret method” (Bio Group Medical System kit; Bio Group Medical System, Talamello (RN), Italy), and the electrophoretic pattern of the serum was assessed using a semi-automated agarose gel electrophoresis system (Sebia Hydrasys®, Norcross, GA, USA). The alanino-aminotransferase (ALT), aspartate-aminotransferase (AST), gamma glutamyl transferase (GGT), triglycerides, cholesterol, uric acid, and creatinine serum concentrations were measured using enzymatic methods in a clinical chemistry analyser (Screen Master Touch, Hospitex diagnostics Srl., Firenze, Italy), as reported by Salamano et al. (2010).

### **7.7.1 Exp 1**

For the Exp 1, alkaline phosphatase (ALP), Ca, P, Mg, and Fe were evaluated on the previously obtained serum using enzymatic methods in a clinical chemistry analyser (Screen Master Touch, Hospitex diagnostics Srl., Firenze, Italy) (Salamano et al., 2010).

Moreover, to obtain plasma the EDTA tubes were centrifugated at 2000 x g for 10 min to separate the cells fractions, and the supernatants were immediately frozen at -80°C and used to determine the antioxidant status and oxidative metabolites. The blood glutathione peroxidase (GPx, EC 1.11.1.9) and total antioxidant status (TAS) activities of the plasma were determined using a Ransel Enzymatic Kit (RS504, Randox Laboratories, Crumlin, UK) and a TAS Colorimetric Kit (NX2332, Randox Laboratories, Crumlin, UK) respectively, according to the manufacturer’s recommendations.

The enzyme immunoassay for the detection and quantification of methylglyoxal (MG) was performed using an OxiSelect™ Methylglyoxal ELISA Kit (STA-811, Cell Biolabs, San Diego, CA, USA), while an OxiSelect™ MDA Adduct Competitive ELISA Kit (STA-832, Cell Biolabs, San Diego, CA, USA) was used for the malondialdehyde (MDA) quantification. Both tests were performed on plasma samples. The 3-

nitrotyrosine plasma concentration was measured by enzyme-linked immunosorbent assay (ELISA) using an OxiSelect™ Nitrotyrosine ELISA Kit (STA-305, Cell Biolabs, San Diego, CA, USA). All the tests were performed according to the manufacturer's instructions.

## **7.8 Slaughtering procedures**

At 50 and 55 days of age (for the Exp 1 and Exp 2, respectively) the final LW of the birds was individually recorded, then 12 ducks per treatment (two birds per pen) were selected on the basis of the average LW and identified with a shank ring. Subsequently, after a feed withdrawal of 12 hours (at 51 days of age), the selected ducks were weighed for the determination of the slaughtering weight (SW) and transferred to a commercial processing plant and slaughtered by electrical stunning and bleeding, according to the standard EU regulations.

The plucked and eviscerated carcasses were obtained, and the head, neck and feet were removed. After the completion of the slaughtering phase, spleen, liver, thymus, bursa of Fabricius, heart, and abdominal fat were removed and weighed, and their weights were expressed as a percentage of the SW.

For caecal microbiota evaluation, caecal content of the slaughtered birds was collected into sterile plastic tubes that were previously refrigerated (for a maximum of 2 h) and frozen at – 80 °C until DNA extraction.

In order to perform the histopathological and histomorphological investigations, samples of organs (spleen, liver, thymus, bursa of Fabricius) and intestinal tracts (duodenum, jejunum and ileum, flushed with 0.9% saline to remove all the contents) were fixed in a 10% buffered formalin solution.

Finally, the hot carcass (HC) weight was recorded, and the HC yields were calculated as a percentage of SW. The carcasses were then stored at +4°C for 24 h for muscle sampling procedures and analyses.

## 7.9 Muscle Sampling

After 24 h of refrigeration at +4°C the carcasses were weighed for the determination of the chilled carcass (CC) weight, and the CC yields were then calculated (%SW). The breast (without skin and ribs) and thighs (with skin and without hipbone) were excised, weighed and collected. The breast and thigh weights were then expressed as percentages of the CC weight.

The pHu and colour were evaluated on the *Pectoralis major* and on the *Biceps femoris* muscles both on the right side. Specifically, the pHu of the *Pectoralis major* and *Biceps femoris* muscles was measured by means of a pH meter (Crison, Crison Instruments, SA, Alella, Spain) equipped with a specific electrode suitable for meat penetration. All the analyses were performed in duplicate. The lightness (L\*), redness (a\*) and yellowness (b\*) colour indexes (Commission International de l'Eclairage, 1976) were measured in the same muscles using a portable Chroma Meter CR-400 Konica Minolta Sensing colorimeter (Minolta Sensing Inc., Osaka, Japan).

### 7.9.1 Exp 1

The left and right thighs were completely deboned to separate the edible meat (used for all the analyses) from the bones.

The left breast and thigh meat were ground using a Grindomix GM 200 device (Retsch GmbH, Haan, Germany); an aliquot was used for FA analysis and the remaining amount was freeze dried, reground and analysed to establish the proximate composition and mineral contents.

Both the right breast and thigh meat were vacuum packaged and stored at -20°C until further analysis to determine the heavy metals and thiobarbituric acid reactive substances (TBARS).

### 7.9.2 Exp 2

The cranial portion of the collected left breasts (approximately 8 x 4 x 3 cm) were weighed, vacuum packaged and then stored at -20°C for the evaluation of the water holding capacity and shear force.

## 7.10 Meat Quality Evaluation

### 7.10.1 Exp 1

Freeze-dried meat samples were used to determine the DM (934.01), ash (967.05), CP (2001.11) and EE (991.36) contents (AOAC, 2000).

The FA composition of the fresh meat from the left breast and thigh was determined according to the method described above for HI meal and for the diets (Christie, 1982). Individual FAMES were expressed as the percentage of the total area of the eluted FAMES.

The atherogenicity (AI) and thrombogenicity (TI) indexes were calculated as described by Ulbricht and Southgate (1991) as follows:

$$AI = [C12:0 + (4 \times C14:0) + C16:0] / (\Sigma MUFA + \Sigma n-6 + \Sigma n-3);$$

$$TI = (C14:0 + C16:0 + C18:0) / [(0.5 \times \Sigma MUFA) + (0.5 \times \Sigma n-6) + (3 \times \Sigma n-3)] + (\Sigma n-3 / \Sigma n-6).$$

Moreover, the Zn, Fe, Mn and Cu contents of the freeze-dried samples were analysed with a double-beam atomic absorption spectrophotometer (AA-7000 Series, Shimadzu Co., Kyoto, Japan), using pooled meat samples from each of the 6 replicate pens (Gresakova et al., 2016). The ERM-BB184 certificate reference materials of the bovine muscle (IRMM, Geel, Belgium) were used to establish the accuracy of the measurements. The samples mineral concentrations were expressed as mg/kg.

In order to perform lipid oxidation evaluation, a total of 10 g of thawed breast and thigh meat samples were analysed by means of a TBARS assay (Witte et al., 1970; Dabbou et al., 2014). Briefly, the thawed meat (10 g) was homogenized and mixed for 30 s with 10% trichloroacetic acid using a Polytron tissue homogenizer (Type PT 10–35; Kinematica GmbH, Luzern, Switzerland), and the supernatant was filtered through Whatman #1 filter paper. One millilitre of filtrate was combined with 1 ml of a 0.02 M aqueous 2-TBA solution, heated in a boiling water bath for 20 min together with a blank containing 1 ml of a TCA/water mix (1/1) and 1 ml of a TBA reagent and subsequently cooled under running tap water (Dabbou et al., 2014). The samples were analysed in duplicate and the absorbance was read at 532 nm with a Helios spectrophotometer (Unicam Limited, Cambridge, UK). The results were expressed as  $\mu\text{g}$  malonaldehyde/g of meat.

The analyses of the trace elements (Co, Cr, Ni, Se), As and the heavy metal (Hg, Cd, Pb) concentrations (see chapter 7.4.1) were performed on 4 breast and thigh meat pools (1 pool per feeding group, each of which was composed of 6 slaughtered animals, 1 bird/pen) using the method describer for HI larva meal (Schiaivone et al., 2019). The results were expressed as mg/kg of sample or mg/kg of sample with 12% humidity.

### **7.10.2 Exp 2**

For the Exp 2, the collected vacuum packaged meat samples from the cranial portion of the left breasts (approximately 65 g) were frozen at -20 °C. For the evaluation of the drip losses, the frozen meat samples were thawed for 24h at 4 °C. Then, samples were blotted for the excess of fluids and weighed for the determination of the drip-loss that were expressed as a percentage of weight lost by the samples during the refrigerated storage period (Petracci and Baéza, 2011). After drip-loss determination, the meat samples were vacuum packaged in plastic bags and cooked by immersion in water bath (80 °C) until their internal temperature was 77 °C (about 15 min). Then the samples were cooled under running cold water for 30 min. For the determination of the cooking losses, the cooled samples were removed from the plastic bags, blotted and weighed. The cooking losses were then expressed as the difference between the sample weight before and after cooking, expressed as a percentage of the thawed weight. A total of four parallelepipedal cores of the cooked meat were obtained from each sample (1 x 1 cm), cutted parallel to muscle fibres. Warner-Bratzler shear force (WBSF) was determined cutting perpendicularly to the longitudinal orientation of the muscle fibres the obtained parallelepipedal cores. For this purpose, a V shaped Warner-Bratzler cutting blade fitted to an Instron 5543 Universal Testing Machine (Instron; Norwood, Massachusetts) was used. The WBSF was measured as the maximum force (kgf/cm<sup>2</sup>) required to shear the parallelepipedal core at a crosshead speed of 200 mm per min.

## **7.11 Histopathological and histomorphological investigations**

Histopathological and histomorphological investigations were performed on the collected organs and gut samples of the slaughtered birds for both the experimental trials.

Spleen, liver, thymus, and bursa of Fabricius samples were collected (0.5-1.5 g/organ) and fixed in a 10% buffered formalin solution, embedded in paraffin wax blocks, sectioned at a thickness of 5 µm, mounted onto glass slides and stained with Haematoxylin & Eosin (HE) for the histopathological examination as reported by Maxie and Miller (2016).

The following histopathological alterations were evaluated: white pulp hyperplasia and depletion in the spleen, cortical depletion in the thymus, follicular depletion and intrafollicular cysts in the bursa of Fabricius, hepatocytes degeneration and lymphoid tissue activation in the liver (Biasato et al., 2016). The observed histopathological alterations were evaluated using a semiquantitative scoring system as follows: absent (score = 0), mild (score = 1), moderate (score = 2) and severe (score = 3). In order to investigate the accumulation of lipids and polysaccharide in the liver, tissue samples of these organs were also stained with Sudan Black (SB) and Periodic acid-Schiff (PAS), respectively. The lipid and polysaccharide staining intensity was scored semi-quantitatively as follows: grade 0 for an absence of staining, grade 1 for mild staining, grade 2 for moderate staining and grade 3 for marked staining. All

the slides were blinded evaluated by three different observers and the discordant cases were reviewed using a multi-head microscope until a unanimous consensus had been reached.

The collected intestine segments were the loop of the duodenum, the tract before Meckel's diverticulum (jejunum) and the tract before the ileocolic junction (ileum). The gut samples were fixed in a 10% buffered formalin solution and prepared for the morphometric analysis using the method described above (Maxie and Miller, 2016). The evaluated morphometric indices were: villus height (Vh, from the tip of the villus to the crypt), crypt depth (Cd, from the base of the villus to the submucosa) and the villus height-to-crypt depth (Vh/Cd) ratio (Laudadio et al 2012). Morphometric analyses were performed on 10 well-oriented and intact villi and 10 crypts chosen from the duodenum, jejunum, and ileum (Qaisrani et al., 2014).

## **7.12 Microbiota evaluation**

The caecal content of the slaughtered birds of both the experimental trials was submitted to DNA extraction and sequencing. Nucleic acid was extracted by caecal samples at each sampling point. Total DNA from the samples was extracted using the RNeasy Power Microbiome KIT (Qiagen, Milan, Italy) following the manufacturer's instructions. One microliters of RNase (Illumina Inc. San Diego. CA) was added to digest RNA in the DNA samples with an incubation of 1 h at 37 °C. The DNA was then quantified using the NanoDrop and standardized at 5 ng/μL.

The DNA directly extracted from caecal samples was used to assess the microbiota diversity by the amplification of the V3-V4 region of the 16S rRNA gene (Klindworth et al., 2013). The PCR products were purified according to the Illumina metagenomic standard procedure (Illumina Inc. San Diego. CA). Sequencing was performed with a MiSeq Illumina instrument with V3 chemistry and generated 250 bp paired end reads according to the manufacturer's instructions.

## 7.13 Statistical Analysis and Bioinformatics

The statistical analyses were performed using the SPSS software package (version 21 for Windows, SPSS Inc., Chicago, IL, USA). Shapiro-Wilk's test established normality or non-normality of distribution. The assumption of equal variances was assessed by means of Levene's homogeneity of variance test. For both the two experimental trials the organs histopathological scores were analysed by means of the Kruskal-Wallis test and Dunn's Multiple Comparison test was used as post-hoc test. Intestinal morphometric indices were analysed in the two trials by fitting a general linear mixed model (GLMM). GLMM allowed the morphometric indices (Vh, Cd and Vh/Cd, separately) to depend on three fixed factors (diet, intestinal segment and interaction between diet and intestinal segment). Animal was included as a random effect to account for repeated measurements in the same duck. The interactions between the levels of the fixed factors were evaluated by means of pairwise comparisons. The results were expressed as the mean and standard error of the means (SEM).

Moreover, the DNA directly extract from caecal samples was used to assess the microbiota by the amplification of the V3-V4 region of the 16S rRNA gene (Klindworth et al., 2013). The PCR products were purified using the Illumina metagenomic standard procedure (Illumina Inc. San Diego. CA). Sequencing was performed with a MiSeq Illumina instrument with V3 chemistry and generated 250 bp paired-end reads according to the manufacturer's instructions. FLASH software (Magoč and Salzberg, 2011) was used to join the reads while QIIME 1.9.0 software (Caporaso et al., 2010) was used for the others step as recently described (Ferrocino et al., 2017). Operational Taxonomic Units (OTUs) were picked at 97% of similarity and taxonomy was assessed by Greengenes16S rRNA gene database v. 2013. OTU table was rarefied at the lowest number of sequence and display the higher taxonomy resolution. Alpha diversity was calculated by the vegan package of R (Dixon, 2003). The diversity indices were further analysed using the pairwise comparisons using Wilcoxon rank sum test to assess differences between the diets. Weighted UniFrac distance matrices and OTU table were used to perform Adonis and Anosim statistical tests in R environment.

### **7.13.1 Exp 1**

In the Exp 1, the mortality rate was analysed by means of a Chi-square test, using the HI0 group as the reference. The experimental unit was the pen for growth performance, digestibility, and meat quality, while the blood traits, the organs histological features, and intestinal morphology were evaluated individually for each duck. The collected data were tested by means of one-way ANOVA. Polynomial contrasts were used to test the linear and quadratic responses to increases in the HI inclusion level in the diet for growth performance, digestibility, blood traits, and meat quality.

Differences among treatments were considered statistically significant when the P values  $\leq 0.05$ .

### **7.13.2 Exp 2**

One-way ANOVA was used to analyse the collected data for growth performance and blood traits. Moreover, one-way ANCOVA (ANOVA with covariate) was conducted to compare the observed behaviours in the experimental treatments using the week as a covariate.

The experimental unit was the pen for growth performance, while the blood traits, the organs histological features, and intestinal morphology were evaluated individually for each duck.

Differences among treatments were considered statistically significant when the P values  $\leq 0.05$ .

## 8. Results

Part of the results of the Exp 1 have already been published in:

- Gariglio M., Dabbou S., Biasato I., Capucchio M.T., Colombino E., Hernández F., Madrid J., Martínez S., Gai F., Caimi C., Bellezza Oddon S., Meneguz M., Trocino A., Vincenzi R., Gasco L., Schiavone A. Nutritional effects of the dietary inclusion of partially defatted *Hermetia illucens* larva meal in Muscovy duck. *Journal of Animal Science and Biotechnology*, 2019, 10:37. doi: 10.1186/s40104-019-0344-7. (IF = 3.441; Q1)
- Gariglio M., Dabbou S., Crispo M., Biasato I., Gai F., Gasco L., Piacente F., Odetti P., Bergagna S., Plachà I., Valle E., Colombino E., Capucchio M.T., Schiavone A. Effects of the dietary inclusion of partially defatted black soldier fly (*Hermetia illucens*) meal on the blood chemistry and tissue (spleen, liver, thymus, and bursa of Fabricius) histology of Muscovy ducks (*Cairina moschata domestica*). *Animals*, 2019, 9, 307. doi:10.3390/ani9060307. (IF = 1.832; Q1)
- 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. Black soldier fly larva in Muscovy duck diets: effects on carcass, meat quality and safety. *Poultry Science*, Under Review.

## 8.1 Exp 1

### 8.1.1 Growth performance

The growth performance results of the Exp 1 are reported in Table 13. The LW and the ADG of the birds were not influenced by the dietary treatments ( $P > 0.05$ ), with an exception observed at 38 days of age, when the HI6 group showed a quadratic response for these two parameters ( $P < 0.05$ ). On the other hand, the DFI and the FCR were not affected by the dietary treatments neither in the different feeding phases or in the whole experimental trial ( $P > 0.05$ ).

**Table 13.** Effect on the growth performance of Muscovy ducks fed diet with increasing HI larva meal levels.

| Items     | Age     | Dietary treatments <sup>1</sup> |         |         |         | SEM   | P-value |           |
|-----------|---------|---------------------------------|---------|---------|---------|-------|---------|-----------|
|           |         | HI0                             | HI3     | HI6     | HI9     |       | Linear  | Quadratic |
| LW (g)    | 3 d     | 70.70                           | 70.41   | 72.65   | 71.51   | 0.60  | 0.405   | 0.733     |
|           | 17 d    | 575.44                          | 567.31  | 572.56  | 575.74  | 4.76  | 0.893   | 0.582     |
|           | 38 d    | 1906.79                         | 1861.96 | 1797.10 | 1900.12 | 14.18 | 0.426   | 0.005     |
|           | 50 d    | 2540.57                         | 2511.14 | 2456.14 | 2554.84 | 20.13 | 0.946   | 0.123     |
| ADG (g/d) | 3-17 d  | 36.05                           | 35.49   | 35.71   | 36.02   | 0.32  | 0.974   | 0.529     |
|           | 18-38 d | 63.40                           | 61.65   | 58.31   | 63.07   | 0.69  | 0.417   | 0.011     |
|           | 39-50 d | 52.81                           | 54.10   | 54.92   | 54.56   | 1.14  | 0.582   | 0.738     |
|           | 3-50 d  | 52.55                           | 51.93   | 50.71   | 52.84   | 0.43  | 0.926   | 0.125     |
| DFI (g/d) | 3-17 d  | 53.69                           | 53.45   | 52.16   | 51.85   | 0.59  | 0.226   | 0.979     |
|           | 18-38 d | 142.03                          | 139.06  | 136.99  | 139.92  | 1.28  | 0.481   | 0.273     |
|           | 39-50 d | 167.48                          | 170.82  | 160.32  | 171.80  | 2.83  | 0.924   | 0.485     |
|           | 3-50 d  | 120.58                          | 121.32  | 117.58  | 121.48  | 1.20  | 0.927   | 0.530     |
| FCR (g/g) | 3-17 d  | 1.49                            | 1.51    | 1.46    | 1.44    | 0.01  | 0.099   | 0.489     |
|           | 18-38 d | 2.24                            | 2.26    | 2.35    | 2.22    | 0.03  | 0.913   | 0.159     |
|           | 39-50 d | 3.17                            | 3.17    | 2.93    | 3.18    | 0.051 | 0.639   | 0.220     |
|           | 3-50 d  | 2.29                            | 2.34    | 2.32    | 2.30    | 0.019 | 0.925   | 0.406     |

Note: HI: *Hermetia illucens*; SEM: standard error of the mean; LW: live weight; ADG: average daily gain; DFI: daily feed intake; FCR: feed conversion ratio. <sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*.

### 8.1.2 Digestibility trial

The ATTDC obtained in the Exp 1 are reported in Table 14. The DM and OM digestibility were not different throughout the trial in all the four experimental treatments ( $P > 0.05$ ). In the starter period the CP digestibility decreased linearly with a minimum corresponding to the HI9 group (3-17 days of age) ( $P < 0.05$ ). On the other hand, EE digestibility showed a linear response in the starter period, with the lowest value observed for HI0 group ( $P < 0.05$ ). In addition, in the grower and finisher periods (from 18 to 38 days of age and from 39 to 50 days of age, respectively) the EE digestibility increased linearly, with the highest values corresponding to the HI9 group ( $P < 0.001$ ).

**Table 14.** Effect of the dietary increasing inclusions of HI larva meal on the nutrients' apparent digestibility of Muscovy ducks.

| Age     | Apparent digestibility | Dietary treatments <sup>1</sup> |       |       |       | SEM   | P-value |           |
|---------|------------------------|---------------------------------|-------|-------|-------|-------|---------|-----------|
|         |                        | HI0                             | HI3   | HI6   | HI9   |       | Linear  | Quadratic |
| 3-17 d  | DM                     | 0.960                           | 0.960 | 0.960 | 0.953 | 0.002 | 0.174   | 0.315     |
|         | CP                     | 0.852                           | 0.872 | 0.828 | 0.817 | 0.007 | 0.010   | 0.195     |
|         | EE                     | 0.945                           | 0.967 | 0.963 | 0.962 | 0.002 | 0.003   | 0.085     |
|         | OM                     | 0.963                           | 0.963 | 0.966 | 0.962 | 0.001 | 0.853   | 0.453     |
| 18-38 d | DM                     | 0.953                           | 0.956 | 0.962 | 0.960 | 0.002 | 0.086   | 0.511     |
|         | CP                     | 0.800                           | 0.802 | 0.802 | 0.828 | 0.005 | 0.085   | 0.269     |
|         | EE                     | 0.958                           | 0.966 | 0.968 | 0.977 | 0.002 | <0.001  | 0.891     |
|         | OM                     | 0.958                           | 0.964 | 0.967 | 0.963 | 0.001 | 0.215   | 0.168     |
| 39-50 d | DM                     | 0.943                           | 0.948 | 0.952 | 0.953 | 0.002 | 0.099   | 0.703     |
|         | CP                     | 0.733                           | 0.682 | 0.715 | 0.718 | 0.012 | 0.913   | 0.259     |
|         | EE                     | 0.953                           | 0.958 | 0.965 | 0.983 | 0.003 | <0.001  | 0.092     |
|         | OM                     | 0.950                           | 0.953 | 0.958 | 0.958 | 0.002 | 0.072   | 0.642     |

Note: HI: *Hermetia illucens*; SEM: standard error of the mean; DM: dry matter; CP: crude protein; EE: ether extract; OM: organic matter. <sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*.

### ***8.1.3 Blood analysis***

The blood analysis results of the Exp 1 are reported in Tables 15 and 16. The haematological traits of the Muscovy ducks fed increasing dietary levels of HI meal were not affected. Serum protein levels were similar among groups, while the triglycerides and cholesterol levels showed a linear decrease to increasing dietary HI larva meal levels ( $P < 0.05$ ; Table 15). The Ca and P serum concentrations were not affected by the dietary treatments. On the contrary, Mg serum concentration showed a linear decrease as a result of the increasing dietary inclusion levels of HI larva meal, while Fe showed a linear increase, with a maximum observed for the HI9 group ( $P < 0.05$ ). AST, ALT, and GGT were not affected by dietary treatments, while ALP decreased linearly, with a minimum for the HI9 group ( $P < 0.05$ ). Moreover, the creatinine values reported a linear decrease with the lowest value observed for the HI9 group ( $P < 0.05$ ; Table 15).

**Table 15.** Effect of the dietary HI larva meal inclusion on the haematological and serum parameters of Muscovy ducks.

| Items                                         | Dietary treatments <sup>1</sup> |        |        |        | SEM   | P-value |           |
|-----------------------------------------------|---------------------------------|--------|--------|--------|-------|---------|-----------|
|                                               | HI0                             | HI3    | HI6    | HI9    |       | Linear  | Quadratic |
| Erythrocytes, 10 <sup>6</sup> (cell/ $\mu$ L) | 5.39                            | 4.98   | 4.98   | 4.92   | 0.11  | 0.160   | 0.429     |
| Leukocytes, 10 <sup>3</sup> (cell/ $\mu$ L)   | 17.58                           | 17.52  | 18.08  | 18.03  | 0.34  | 0.547   | 0.991     |
| Heterophils (%)                               | 50.83                           | 51.75  | 46.18  | 51.16  | 1.31  | 0.697   | 0.443     |
| Lymphocytes (%)                               | 47.75                           | 46.17  | 50.91  | 46.58  | 1.36  | 0.920   | 0.623     |
| Monocytes (%)                                 | 0.58                            | 1.00   | 1.09   | 0.83   | 0.14  | 0.501   | 0.235     |
| Eosinophils (%)                               | 0.33                            | 0.75   | 1.18   | 1.17   | 0.19  | 0.092   | 0.576     |
| Basophils (%)                                 | 0.50                            | 0.33   | 0.64   | 0.25   | 0.12  | 0.673   | 0.646     |
| H/L                                           | 0.98                            | 0.96   | 0.96   | 0.97   | 0.03  | 0.997   | 0.876     |
| Total Protein (g/dl)                          | 4.26                            | 4.84   | 4.99   | 4.79   | 0.11  | 0.086   | 0.082     |
| Triglycerides (mg/dl)                         | 73.27                           | 58.38  | 55.93  | 51.12  | 3.08  | 0.012   | 0.395     |
| Cholesterol (mg/dl)                           | 90.79                           | 85.25  | 82.71  | 69.13  | 3.13  | 0.016   | 0.507     |
| Ca (mg/dl)                                    | 10.02                           | 10.59  | 10.56  | 9.27   | 0.37  | 0.469   | 0.840     |
| P (mg/dl)                                     | 3.87                            | 4.35   | 3.99   | 3.93   | 0.16  | 0.900   | 0.407     |
| Mg (mg/dl)                                    | 1.48                            | 1.43   | 1.32   | 1.25   | 0.03  | 0.002   | 0.858     |
| Fe (mg/l)                                     | 310.22                          | 327.33 | 371.18 | 406.39 | 13.80 | 0.007   | 0.731     |
| AST (U/l)                                     | 27.14                           | 27.32  | 27.08  | 28.58  | 0.89  | 0.622   | 0.717     |
| ALT (U/l)                                     | 27.54                           | 30.49  | 29.48  | 25.41  | 1.30  | 0.530   | 0.187     |
| GGT (U/l)                                     | 4.83                            | 4.23   | 4.82   | 4.92   | 0.23  | 0.685   | 0.452     |
| ALP (U/l)                                     | 2003.6                          | 1889.8 | 1876.4 | 1831.1 | 22.59 | 0.008   | 0.426     |
| Uric acid (mg/dl)                             | 3.98                            | 3.88   | 3.69   | 3.31   | 0.17  | 0.152   | 0.679     |
| Creatinine (mg/dl)                            | 0.30                            | 0.29   | 0.29   | 0.25   | 0.01  | 0.022   | 0.526     |

Note: HI: *Hermetia illucens*; SEM: standard error of the mean; H/L: heterophils to lymphocytes ratio; AST: aspartate-aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transferase; ALP: alkaline phosphatase. <sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*.

The results of the antioxidant enzymes and oxidative metabolites investigation are reported in Table 16. The GPx, TAS and MG levels were similar between groups, while MDA and nitrotyrosine showed a linear decrease with increasing dietary levels of HI larva meal ( $P < 0.05$ ).

**Table 16.** Antioxidant enzymes and oxidative metabolites of the Muscovy ducks fed increasing levels of HI larva meal.

| Items                   | Dietary treatments <sup>1</sup> |        |        |        | SEM   | P-value |           |
|-------------------------|---------------------------------|--------|--------|--------|-------|---------|-----------|
|                         | HI0                             | HI3    | HI6    | HI9    |       | Linear  | Quadratic |
| GPx (U/g Hb)            | 216.54                          | 240.27 | 229.74 | 246.39 | 7.51  | 0.250   | 0.816     |
| TAS (mmol/l)            | 1.48                            | 1.47   | 1.46   | 1.43   | 0.03  | 0.618   | 0.870     |
| MG ( $\mu\text{g/ml}$ ) | 0.49                            | 0.50   | 0.47   | 0.46   | 0.01  | 0.307   | 0.747     |
| MDA (pmol/ml)           | 161.99                          | 169.97 | 148.73 | 147.10 | 2.10  | 0.000   | 0.152     |
| Nitrotyrosine (nM)      | 193.10                          | 133.42 | 122.66 | 78.67  | 11.01 | 0.000   | 0.677     |

Note: HI: *Hermetia illucens*; SEM: standard error of the mean; GPx: glutathione peroxidase; Hb: haemoglobin; TAS: total antioxidant status; MG: methylglyoxal; MDA: malondialdehyde. <sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*.

### 8.1.4 Slaughtering performance

The slaughtering performance results of the Exp 1 are reported in Table 17. The SW, and consequently the HC and CC weights, showed a quadratic response with a minimum observed for the HI6 group ( $P < 0.05$ ). Despite this, the HC and CC yields were not influenced by the dietary treatments. The spleen, liver, bursa of Fabricius and heart yields (expressed as a percentage of the SW) were similar among groups. On the other hand, the abdominal fat showed a quadratic response, showing the minimum value for the HI6 group ( $P < 0.05$ ; Table 17).

**Table 17.** Slaughtering performance of Muscovy ducks fed increasing partially defatted HI larva meal.

| Items                      | Dietary treatments <sup>1</sup> |         |         |         | SEM   | P-value |           |
|----------------------------|---------------------------------|---------|---------|---------|-------|---------|-----------|
|                            | HI0                             | HI3     | HI6     | HI9     |       | Linear  | Quadratic |
| SW (g)                     | 2491.83                         | 2407.66 | 2373.67 | 2487.00 | 16.67 | 0.727   | 0.003     |
| HC weight (g)              | 1606.31                         | 1567.92 | 1550.57 | 1630.72 | 10.90 | 0.544   | 0.006     |
| CC weight (g)              | 1576.55                         | 1534.12 | 1521.45 | 1597.62 | 10.72 | 0.577   | 0.005     |
| HC yield (% SW)            | 64.47                           | 65.12   | 65.38   | 65.58   | 0.25  | 0.115   | 0.655     |
| CC yield (% SW)            | 63.30                           | 63.72   | 64.14   | 64.24   | 0.23  | 0.123   | 0.734     |
| Breast yield (% CC weight) | 20.00                           | 20.52   | 20.03   | 20.58   | 0.24  | 0.575   | 0.977     |
| Thigh yield (% CC weight)  | 29.44                           | 29.56   | 30.15   | 29.58   | 0.21  | 0.657   | 0.403     |
| Spleen (% SW)              | 0.08                            | 0.07    | 0.08    | 0.09    | 0.00  | 0.831   | 0.157     |
| Liver (% SW)               | 1.88                            | 1.87    | 1.87    | 1.75    | 0.03  | 0.127   | 0.387     |
| Bursa of Fabricius (% SW)  | 0.12                            | 0.15    | 0.13    | 0.15    | 0.00  | 0.289   | 0.668     |
| Heart (% SW)               | 0.61                            | 0.60    | 0.58    | 0.58    | 0.01  | 0.247   | 0.623     |
| Abdominal fat (% SW)       | 2.31                            | 2.01    | 1.98    | 2.23    | 0.06  | 0.647   | 0.030     |

Note: HI: *Hermetia illucens*; SEM: standard error of the mean; SW: slaughtering weight; HC: hot carcass; CC: chilled carcass. <sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*.

### 8.1.5 Meat quality evaluation

The results for pHu, colour, proximate and mineral compositions and TBARS of breast and leg meat (BM and LM, respectively) of the Exp 1 are reported in Table 18.

**Table 18.** pHu and colour, proximate composition, mineral profile, and lipid oxidation (TBARS,  $\mu\text{g}$  MDA/g fresh meat) of the breast meat (BM) and leg meat (LM) of Muscovy ducks fed increasing partially defatted HI larva meal.

| Items             |    | Dietary treatments <sup>1</sup> |      |      |      | SEM  | P-value |           |
|-------------------|----|---------------------------------|------|------|------|------|---------|-----------|
|                   |    | HI0                             | HI3  | HI6  | HI9  |      | Linear  | Quadratic |
| pHu               | BM | 5.88                            | 5.86 | 5.84 | 5.83 | 0.02 | 0.401   | 0.860     |
|                   | LM | 5.92                            | 5.89 | 5.95 | 5.90 | 0.02 | 0.976   | 0.778     |
| L*                | BM | 45.6                            | 46.1 | 46.9 | 45.9 | 0.42 | 0.683   | 0.374     |
|                   | LM | 44.4                            | 46.1 | 46.3 | 45.1 | 0.45 | 0.528   | 0.116     |
| a*                | BM | 13.1                            | 12.5 | 12.6 | 13.7 | 0.34 | 0.566   | 0.232     |
|                   | LM | 11.4                            | 11.7 | 10.3 | 11.2 | 0.33 | 0.549   | 0.682     |
| b*                | BM | 11.3                            | 11.1 | 11.3 | 10.8 | 0.27 | 0.549   | 0.719     |
|                   | LM | 10.7                            | 11.3 | 10.1 | 10.3 | 0.20 | 0.190   | 0.542     |
| Moisture (%)      | BM | 77.2                            | 77.1 | 77.2 | 77.2 | 0.06 | 0.629   | 0.808     |
|                   | LM | 74.4                            | 74.8 | 74.6 | 74.8 | 0.09 | 0.250   | 0.697     |
| Protein (%)       | BM | 20.1                            | 20.2 | 20.1 | 20.1 | 0.06 | 0.803   | 0.374     |
|                   | LM | 21.0                            | 20.8 | 21.0 | 21.0 | 0.06 | 0.691   | 0.346     |
| Ether extract (%) | BM | 0.88                            | 0.91 | 0.85 | 0.87 | 0.02 | 0.412   | 0.823     |
|                   | LM | 2.57                            | 2.48 | 2.74 | 2.48 | 0.09 | 0.991   | 0.638     |
| Ash (%)           | BM | 1.38                            | 1.35 | 1.35 | 1.36 | 0.01 | 0.523   | 0.259     |
|                   | LM | 1.18                            | 1.16 | 1.17 | 1.17 | 0.00 | 0.682   | 0.422     |
| Zn (mg/kg)        | BM | 10.6                            | 10.5 | 10.3 | 10.3 | 0.09 | 0.281   | 0.675     |
|                   | LM | 20.5                            | 21.2 | 21.6 | 21.2 | 0.26 | 0.308   | 0.337     |
| Fe (mg/kg)        | BM | 30.5                            | 29.8 | 27.4 | 29.1 | 0.54 | 0.186   | 0.266     |
|                   | LM | 13.8                            | 14.1 | 13.1 | 12.7 | 0.30 | 0.108   | 0.546     |
| Mn (mg/kg)        | BM | 0.16                            | 0.13 | 0.13 | 0.14 | 0.00 | 0.103   | 0.055     |
|                   | LM | 0.11                            | 0.10 | 0.11 | 0.11 | 0.00 | 0.276   | 0.391     |
| Cu (mg/kg)        | BM | 2.75                            | 3.02 | 3.03 | 3.01 | 0.07 | 0.209   | 0.288     |
|                   | LM | 1.06                            | 1.16 | 1.23 | 1.31 | 0.03 | <0.001  | 0.814     |
| TBARS             | BM | 2.82                            | 2.75 | 2.75 | 2.69 | 0.09 | 0.645   | 0.969     |
|                   | LM | 1.36                            | 1.19 | 2.23 | 1.16 | 0.04 | 0.118   | 0.493     |

Note: HI: *Hermetia illucens*; SEM: standard error of the mean; pHu: ultimate pH; L\*: lightness; a\*: redness; b\*: yellowness; TBARS: thiobarbituric acid-reactive substances. <sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*.

The pHu and the colour of the BM and LM, as well as their proximate and mineral compositions, and the TBARS values, were not different between the dietary treatments ( $P > 0.05$ ). For what the minerals levels concern, the thigh Cu content showed a linear increase, with a maximum for the HI9 group ( $P < 0.001$ ).

Table 19 depicts the BM and LM FA profiles of the Exp 1. The lauric (C12:0) and myristic (C14:0) acids showed a linear increase accordingly to the HI meal inclusion levels in the diet, with the highest value observed in the HI9 group ( $P < 0.001$ ), in both the BM and in the LM. On the other hand, palmitic (C16:0) acid decreased linearly in the thigh meat ( $P < 0.05$ ). Nevertheless, the SFA content of the BM resulted not affected by the dietary HI larva inclusion levels. Contrarily, a linear increase was observed for SFA content in the LM, with the highest value observed for the HI9 group ( $P < 0.05$ ). Among the MUFAs, palmitoleic (C16:1 n-7) acid showed a linear decrease in the LM, with a minimum for the HI9 group ( $P < 0.05$ ). Moreover, the  $\alpha$ -linolenic (C18:3 n-3) acid showed a linear and quadratic response in the BM, with a minimum corresponding to the HI9 group ( $P < 0.05$ ). On the contrary, the  $\alpha$ -linolenic acid levels increased linearly in the LM according to the increasing dietary levels of HI larva meal (maximum for the HI9 group;  $P < 0.001$ ). Furthermore, quadratic response was observed for the docosahexaenoic (DHA, C22:6 n-3) acid ( $P < 0.05$ ), and a linear response was observed for docosapentaenoic n-6 (C22:5 n-6) acid in the BM, with the highest value observed for the control group ( $P < 0.05$ ). The  $\sum n-6/\sum n-3$  ratio showed a linear decrease both in BM and LM ( $P < 0.05$ ).

Finally, the lowest AI values for the BM and LM were observed for the control group ( $P < 0.05$ ), where linear responses were observed.

**Table 19.** Fatty acid composition (% of total FAME) of the breast meat (BM) and leg meat (LM) of Muscovy ducks fed increasing partially defatted HI larva meal.

| Fatty acids        |    | Dietary treatments <sup>1</sup> |       |       |       | SEM  | P-value |           |
|--------------------|----|---------------------------------|-------|-------|-------|------|---------|-----------|
|                    |    | HI0                             | HI3   | HI6   | HI9   |      | Linear  | Quadratic |
| C12:0              | BM | 0.12                            | 0.34  | 0.54  | 0.80  | 0.04 | <0.001  | 0.610     |
|                    | LM | 0.13                            | 0.60  | 1.19  | 1.66  | 0.09 | <0.001  | 0.975     |
| C14:0              | BM | 0.37                            | 0.44  | 0.52  | 0.57  | 0.01 | <0.001  | 0.634     |
|                    | LM | 0.49                            | 0.57  | 0.74  | 0.78  | 0.02 | <0.001  | 0.360     |
| C16:0              | BM | 20.62                           | 21.11 | 20.93 | 20.48 | 0.14 | 0.650   | 0.101     |
|                    | LM | 20.43                           | 20.02 | 20.42 | 19.45 | 0.12 | 0.018   | 0.232     |
| C18:0              | BM | 12.97                           | 11.72 | 12.16 | 12.02 | 0.17 | 0.101   | 0.088     |
|                    | LM | 9.25                            | 8.94  | 8.63  | 9.12  | 0.12 | 0.529   | 0.112     |
| C16:1 n-7          | BM | 1.18                            | 1.31  | 1.20  | 1.18  | 0.03 | 0.720   | 0.178     |
|                    | LM | 2.05                            | 1.96  | 1.97  | 1.83  | 0.03 | 0.039   | 0.661     |
| C18:1 n-9          | BM | 23.58                           | 25.77 | 25.10 | 25.48 | 0.31 | 0.063   | 0.133     |
|                    | LM | 29.69                           | 29.80 | 29.88 | 29.52 | 0.24 | 0.850   | 0.643     |
| C18:1 n-7          | BM | 2.35                            | 2.17  | 2.20  | 2.23  | 0.03 | 0.345   | 0.144     |
|                    | LM | 1.89                            | 1.84  | 1.80  | 1.82  | 0.02 | 0.298   | 0.463     |
| C18:2 n-6          | BM | 21.07                           | 21.92 | 21.28 | 21.76 | 0.18 | 0.367   | 0.603     |
|                    | LM | 24.03                           | 24.73 | 24.33 | 24.23 | 0.20 | 0.904   | 0.319     |
| C18:3 n-3          | BM | 1.17                            | 1.15  | 1.16  | 1.11  | 0.02 | 0.008   | 0.046     |
|                    | LM | 1.53                            | 1.61  | 1.73  | 1.71  | 0.02 | <0.001  | 0.158     |
| C20:2 n-6          | BM | 0.51                            | 0.43  | 0.47  | 0.46  | 0.01 | 0.257   | 0.180     |
|                    | LM | 0.22                            | 0.22  | 0.21  | 0.22  | 0.00 | 0.787   | 0.780     |
| C22:5 n-6          | BM | 1.99                            | 0.73  | 0.82  | 0.77  | 0.03 | 0.026   | 0.080     |
|                    | LM | 0.73                            | 0.64  | 0.61  | 0.67  | 0.02 | 0.359   | 0.133     |
| C20:5 n-3 (EPA)    | BM | 0.16                            | 0.14  | 0.16  | 0.17  | 0.00 | 0.449   | 0.154     |
|                    | LM | 0.12                            | 0.11  | 0.10  | 0.12  | 0.00 | 0.933   | 0.218     |
| C22:5 n-3 (DPA)    | BM | 0.72                            | 0.57  | 0.64  | 0.60  | 0.02 | 0.112   | 0.172     |
|                    | LM | 0.50                            | 0.46  | 0.44  | 0.46  | 0.01 | 0.263   | 0.270     |
| C22:6 n-3 (DHA)    | BM | 0.67                            | 0.52  | 0.58  | 0.58  | 0.02 | 0.217   | 0.041     |
|                    | LM | 0.50                            | 0.45  | 0.41  | 0.47  | 0.02 | 0.385   | 0.110     |
| ΣSFA <sup>2</sup>  | BM | 34.59                           | 34.10 | 34.68 | 34.42 | 0.11 | 0.958   | 0.600     |
|                    | LM | 30.54                           | 30.38 | 31.24 | 31.29 | 0.11 | 0.001   | 0.601     |
| ΣMUFA <sup>2</sup> | BM | 28.16                           | 30.30 | 29.57 | 30.00 | 0.32 | 0.086   | 0.170     |
|                    | LM | 34.51                           | 34.08 | 34.16 | 33.67 | 0.26 | 0.314   | 0.956     |
| ΣPUFA <sup>2</sup> | BM | 36.35                           | 34.77 | 34.87 | 34.69 | 0.32 | 0.092   | 0.277     |
|                    | LM | 34.15                           | 34.38 | 33.46 | 33.87 | 0.26 | 0.457   | 0.860     |
| Σn-3 <sup>2</sup>  | BM | 2.60                            | 2.47  | 2.61  | 2.59  | 0.03 | 0.637   | 0.312     |
|                    | LM | 2.66                            | 2.64  | 2.70  | 2.77  | 0.03 | 0.173   | 0.516     |
| Σn-6 <sup>2</sup>  | BM | 33.54                           | 32.10 | 32.05 | 31.87 | 0.30 | 0.059   | 0.287     |
|                    | LM | 31.28                           | 31.53 | 30.54 | 30.90 | 0.23 | 0.319   | 0.918     |

Table 19. *Continue*

| Fatty acids              |    | Dietary treatments <sup>1</sup> |       |       |       | SEM  | P-value |           |
|--------------------------|----|---------------------------------|-------|-------|-------|------|---------|-----------|
|                          |    | HI0                             | HI3   | HI6   | HI9   |      | Linear  | Quadratic |
| $\Sigma n-6/\Sigma n-3$  | BM | 12.93                           | 13.04 | 12.30 | 12.31 | 0.09 | <0.001  | 0.742     |
|                          | LM | 11.80                           | 11.96 | 11.35 | 11.19 | 0.08 | 0.001   | 0.299     |
| $\Sigma PUFA/\Sigma SFA$ | BM | 1.05                            | 1.02  | 1.01  | 1.01  | 0.10 | 0.137   | 0.469     |
|                          | LM | 1.12                            | 1.13  | 1.07  | 1.08  | 0.01 | 0.051   | 0.948     |
| AI                       | BM | 0.34                            | 0.36  | 0.38  | 0.36  | 0.00 | 0.020   | 0.281     |
|                          | LM | 0.33                            | 0.33  | 0.36  | 0.36  | 0.00 | <0.001  | 0.268     |
| TI                       | BM | 0.96                            | 0.94  | 0.95  | 0.93  | 0.00 | 0.240   | 0.983     |
|                          | LM | 0.82                            | 0.81  | 0.82  | 0.81  | 0.00 | 0.631   | 0.894     |

Note: HI: *Hermetia illucens*; SEM: standard error of the mean; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; AI: atherogenicity index; TI: thrombogenicity index. <sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*.

<sup>2</sup>Including minor FA.

Table 20 depicts the BM and LM concentrations of trace elements, As and heavy metals. The As, Hg, Cd and Pb concentrations (on average) were below 0.01 mg/kg 12% humidity in both the BM and LM and below the EU limits reported for chicken meat (EC, 2006).

**Table 20.** Trace elements, As, and heavy metal content in the breast meat (BM) and leg meat (LM) of Muscovy ducks fed increasing levels of HI meal (one pool per feeding group).

| Items            |    | Dietary treatments <sup>1</sup> |       |       |       | MRL<br>(Regulation 881/2006/EC) |
|------------------|----|---------------------------------|-------|-------|-------|---------------------------------|
|                  |    | HI0                             | HI3   | HI6   | HI9   |                                 |
| Co (mg/kg)       | BM | ≤0.05                           | ≤0.05 | ≤0.05 | ≤0.05 | Not legislated                  |
|                  | LM | ≤0.05                           | ≤0.05 | ≤0.05 | ≤0.05 |                                 |
| Cr (mg/kg)       | BM | 0.09                            | 0.33  | 0.52  | 0.27  | Not legislated                  |
|                  | LM | 0.05                            | 0.07  | ≤0.05 | 0.06  |                                 |
| Ni (mg/kg)       | BM | 0.11                            | 0.22  | 0.28  | 0.16  | Not legislated                  |
|                  | LM | ≤0.05                           | 0.07  | 0.06  | 0.18  |                                 |
| Se (mg/kg)       | BM | 0.26                            | ≤0.25 | 0.30  | 0.29  | Not legislated                  |
|                  | LM | ≤0.25                           | ≤0.25 | ≤0.25 | ≤0.25 |                                 |
| As (mg/kg 12% h) | BM | ≤0.01                           | 0.013 | ≤0.01 | ≤0.01 | Not legislated                  |
|                  | LM | ≤0.01                           | ≤0.01 | ≤0.01 | ≤0.01 |                                 |
| Hg (mg/kg 12% h) | BM | ≤0.01                           | ≤0.01 | ≤0.01 | ≤0.01 | Not legislated                  |
|                  | LM | ≤0.01                           | ≤0.01 | ≤0.01 | ≤0.01 |                                 |
| Cd (mg/kg 12% h) | BM | ≤0.01                           | ≤0.01 | ≤0.01 | ≤0.01 | 0.050 (EU n. 488/14)            |
|                  | LM | ≤0.01                           | ≤0.01 | ≤0.01 | ≤0.01 |                                 |
| Pb (mg/kg 12% h) | BM | 0.02                            | ≤0.01 | ≤0.01 | ≤0.01 | 0.10 (EU n. 1005/15)            |
|                  | LM | ≤0.01                           | ≤0.01 | ≤0.01 | ≤0.01 |                                 |

Note: HI: *Hermetia illucens*; MRL: Maximum Residue Limit; mg/kg 12% h: mg/kg 12% humidity. <sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*.

### 8.1.6 Histopathological and histomorphological investigations

The results of the organs histopathological investigation and intestinal histomorphological evaluations of the Exp 1 are reported in Tables 21 and 22, respectively. No macroscopic lesions were observed in any of the slaughtered birds during the anatomopathological examination. Regardless of the dietary treatment, the spleen showed mild, multifocal white pulp hyperplasia (Table 21), while the thymus showed mild, multifocal cortical depletion. Furthermore, the bursa of Fabricius showed mild to moderate, multifocal to diffuse follicular depletion. The liver showed mild to severe, multifocal to diffuse steatosis or vacuolar degeneration of the hepatocytes. The severity of the observed histopathological changes was not affected by the dietary HI larva meal inclusion ( $P > 0.05$ , Table 21).

**Table 21.** Histopathological scores of the Muscovy ducks fed increasing HI larva meal.

| Items              | Dietary treatments <sup>1</sup> |      |      |      | SEM  | P-value |
|--------------------|---------------------------------|------|------|------|------|---------|
|                    | HI0                             | HI3  | HI6  | HI9  |      |         |
| Spleen             | 0.42                            | 0.29 | 0.27 | 0.20 | 0.07 | 0.740   |
| Liver              | 1.38                            | 1.88 | 0.77 | 1.38 | 0.15 | 0.096   |
| Thymus             | 0.10                            | 0.18 | 0.09 | 0.00 | 0.06 | 0.549   |
| Bursa of Fabricius | 0.33                            | 0.25 | 0.00 | 0.36 | 0.07 | 0.224   |

Note: HI: *Hermetia illucens*; SEM: standard error of the mean. <sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*.

Regarding the histomorphological investigation, the Vh, Cd and Vh/Cd were affected by the intestinal segment ( $P < 0.001$ ). However, no effects of the diet or interaction between the diet and the intestinal segment ( $P > 0.05$ ) were observed on the morphometric indices of the slaughtered birds. The duodenum showed higher Vh and Cd values ( $P < 0.05$  and  $P < 0.01$ , respectively) than the ileum, and the morphometric indices were also greater ( $P < 0.05$ ) in the jejunum than in the ileum. Moreover, the duodenum showed a greater Vh/Cd ( $P < 0.001$ ) than the other gut segments (Table 22).

**Table 22.** Effects of diet, intestinal segment and interaction between diet and intestinal segment on the intestinal morphometric indices of the Muscovy ducks.

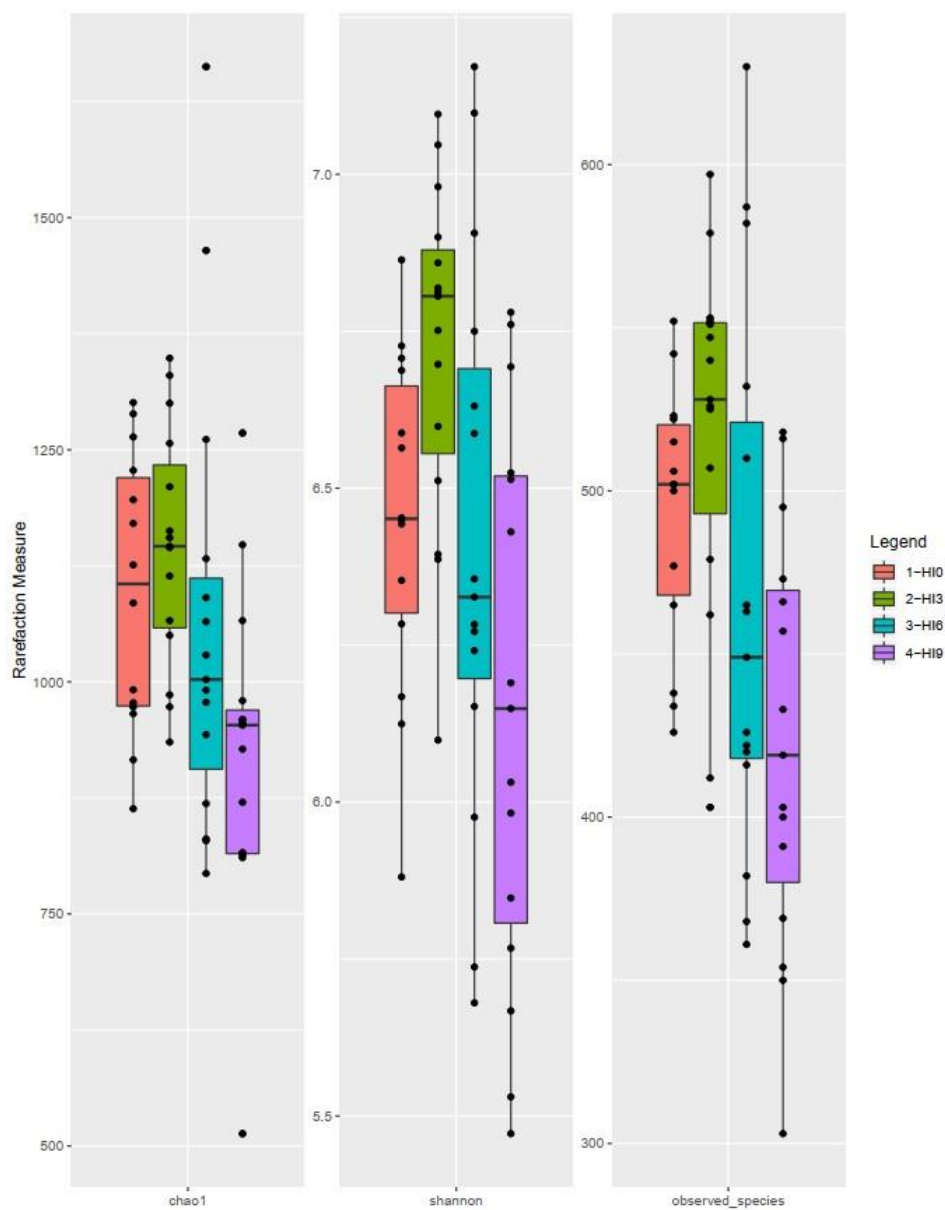
| Index   | Diet (D) <sup>1</sup> |       |       |       | Intestinal segment (IS) <sup>2</sup> |                   |                   | SEM  |      | P-value |        |        |
|---------|-----------------------|-------|-------|-------|--------------------------------------|-------------------|-------------------|------|------|---------|--------|--------|
|         | HI0                   | HI3   | HI16  | HI19  | DU                                   | JE                | IL                | D    | IS   | D       | IS     | D × IS |
| Vh (mm) | 1.55                  | 1.51  | 1.52  | 1.63  | 2.12 <sup>a</sup>                    | 1.41 <sup>b</sup> | 1.14 <sup>c</sup> | 0.06 | 0.06 | 0.442   | <0.001 | 0.508  |
| Cd (mm) | 0.16                  | 0.14  | 0.15  | 0.15  | 0.18 <sup>a</sup>                    | 0.15 <sup>b</sup> | 0.13 <sup>c</sup> | 0.01 | 0.01 | 0.346   | <0.001 | 0.782  |
| Vh/Cd   | 9.80                  | 11.06 | 10.67 | 10.83 | 12.62 <sup>a</sup>                   | 9.67 <sup>b</sup> | 9.48 <sup>b</sup> | 0.60 | 0.45 | 0.469   | <0.001 | 0.966  |

Note: HI: *Hermetia illucens*; Vh villus height; Cd crypt depth; Vh/Cd villus height-to-crypt depth ratio. <sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*. <sup>2</sup>Three intestinal segments: DU = duodenum, JE = jejunum, IL = ileum.

### 8.1.7 Microbiota

After sequencing and quality filtering 740739 reads were obtained and used for the further analysis with an average value of 12143 reads/sample. Analysis of rarefactions and estimated sample coverage indicated that there was satisfactory coverage of all samples (median coverage value of 95%). Comparing the alpha diversity measures (Shannon and Chao1 indexes) of the Exp 1, a significant increase in the diversity values in HI3 group was observed, while a decrease was observed in HI6 and HI9 groups [false discovery rate (FDR) < 0.05, Figure 8].

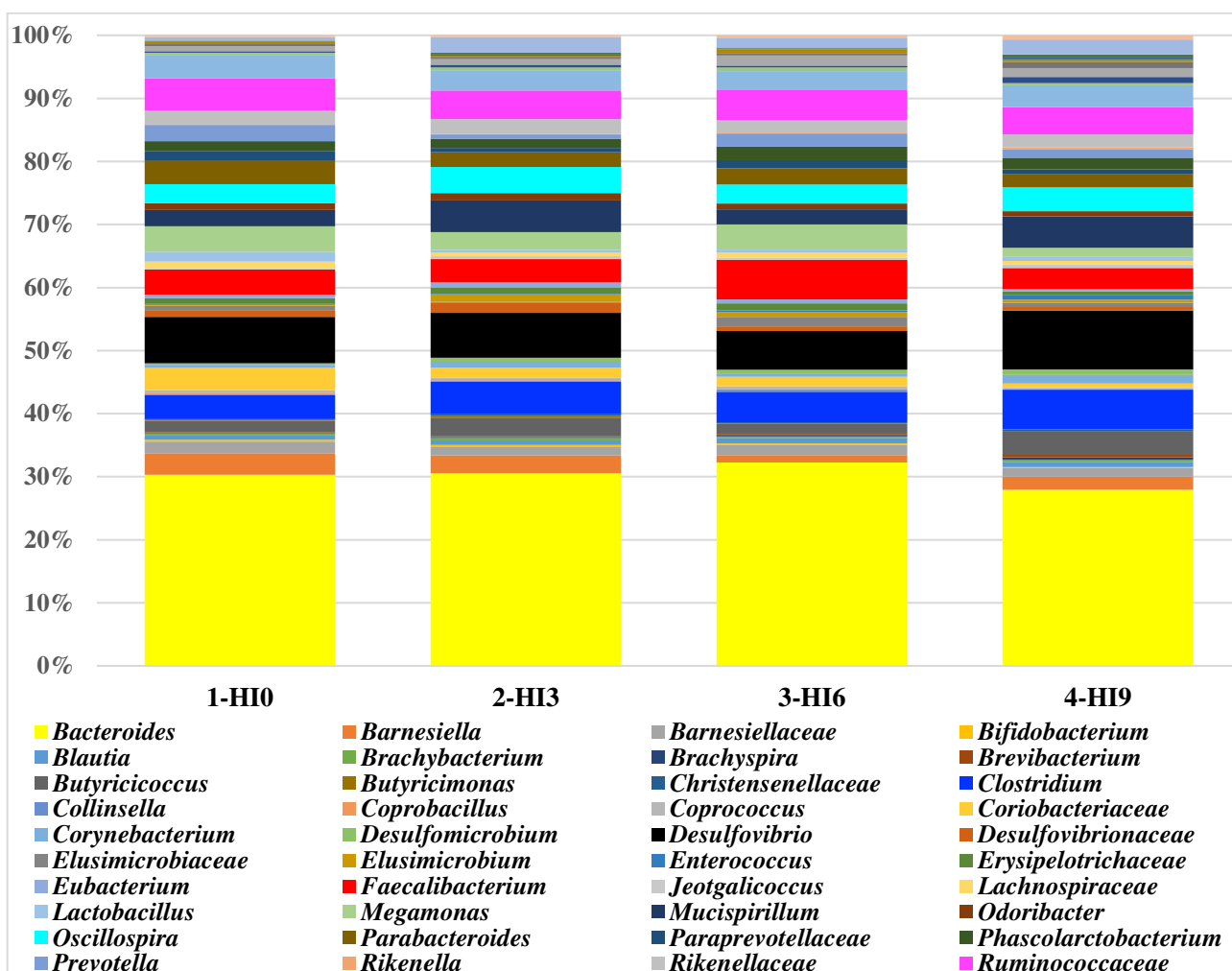
**Figure 8.** Alpha diversity measures (Chao1, Shannon and observed species indexes) of ceecal microbiota of Muscovy duck fed increasing dietary levels of HI larva meal<sup>1</sup>.



Note: HI: *Hermetia illucens*. <sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*.

The microbiota composition resulted similar between the four dietary treatments (Figure 9). The caecal microbial composition of the Muscovy ducks fed increasing dietary levels of HI larva meal was mainly characterized by *Bacteroides* (about 30% of the relative abundance) followed by *Desulfovibrio* (mean value of 8%) and *Ruminococcaceae* (mean value of 4%). The control group showed higher amounts of *Coriobacteriaceae* and *Parabacteroides* (relative abundance about 3%), while in HI9 group a reduction in *Coriobacteriaceae* and *Parabacteroides* was observed, with a relative abundance of 0.5% and 1.7%, respectively.

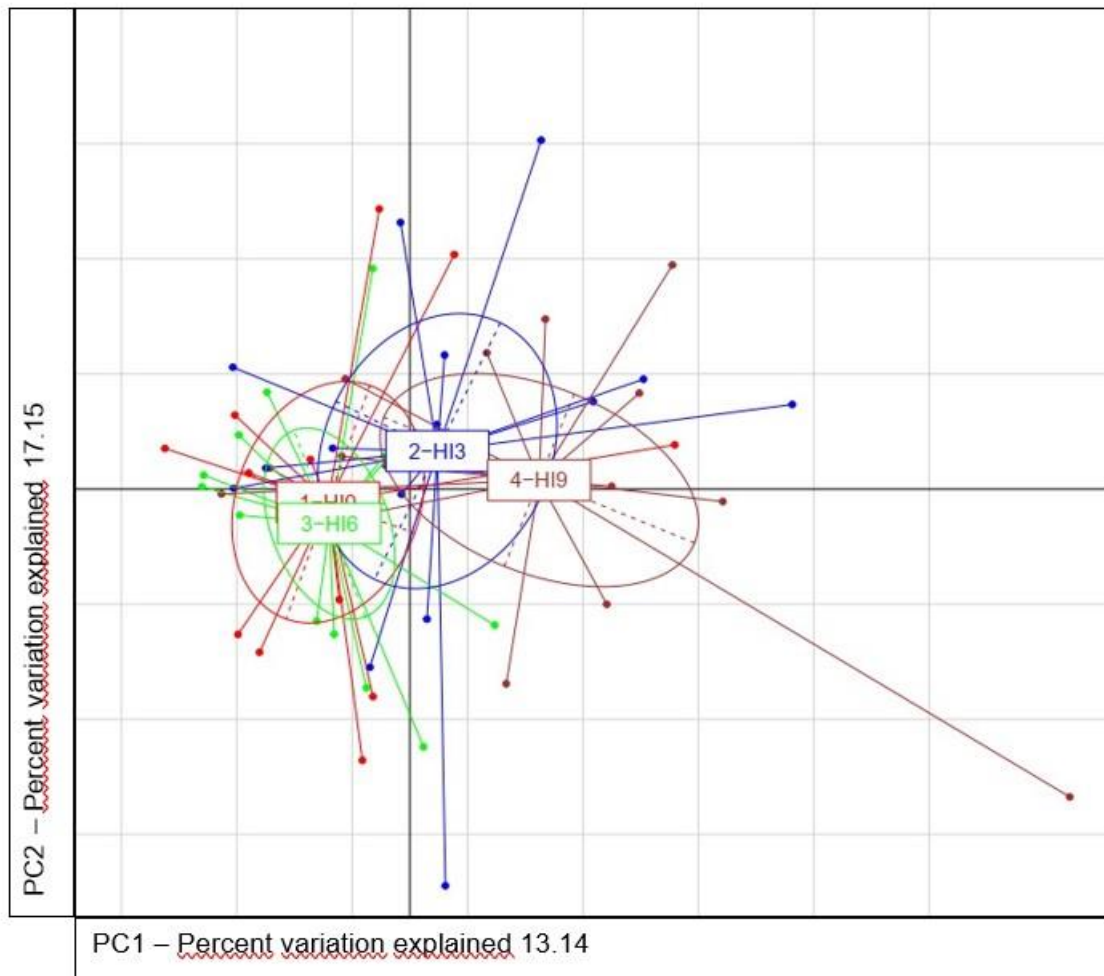
**Figure 9.** Relative abundance of the main bacterial families and genera in caecal samples of Muscovy ducks fed increasing dietary levels of HI larva meal<sup>1</sup>.



Note: HI: *Hermetia illucens*. <sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*.

The Principal Component Analysis (PCA), that is based on the relative abundance of the main OTUs (Figure 10), highlighted a not clear separation of the microbial membership between groups, but only a certain degree of separation. More deeply, the control and HI6 groups were found to be the most similar and clustered together, while HI3 and HI9 groups deviate slightly from the other two groups. However, it is possible to observe a microbial signature due to the dietary HI larva meal inclusion.

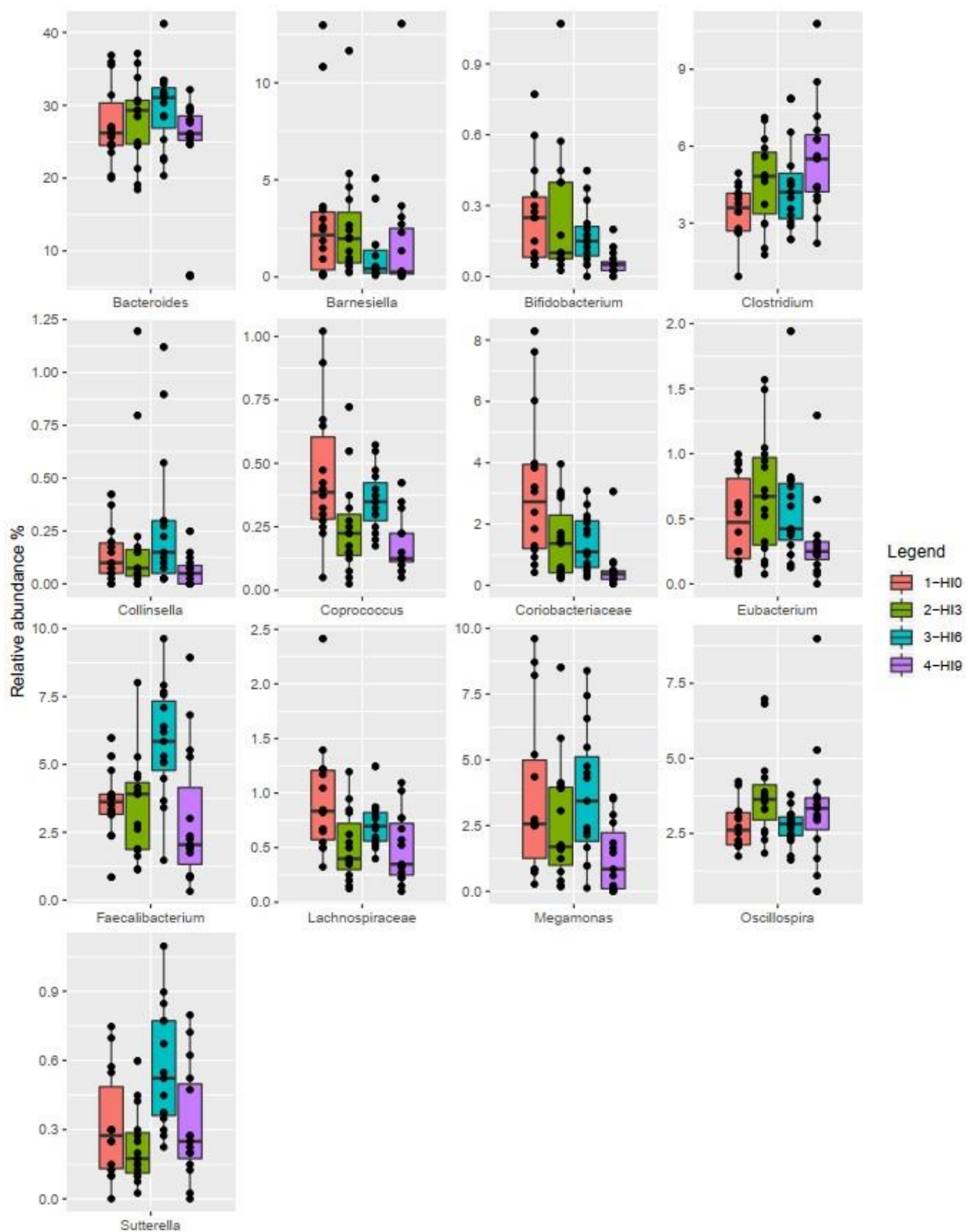
**Figure 10.** Bacterial community composition (weighted UniFrac beta diversity, PCA plots) in caecal samples of Muscovy ducks fed increasing dietary levels of HI larva meal<sup>1</sup>.



Note: HI: *Hermetia illucens*. <sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*.

At a genus level, the *Clostridium* showed the higher abundance in the HI9 group (FDR < 0.05). On the other hand, the HI6 group was characterized by the presence of *Sutterella*, *Faecalibacterium* and *Megamonas*, while *Coprococcus* and *Lachnospiraceae* were associated to the control and the HI6 groups. Moreover, in the HI6 group *Collinsella* resulted more prevalent, while *Barnesiella* was reduced compared to the other groups (FDR < 0.05, Figure 11).

**Figure 11.** Boxplots of *Bacteroides*, *Barnesiella*, *Bifidobacterium*, *Clostridium*, *Collinsella*, *Coprococcus*, *Coriobacteriaceae*, *Eubacterium*, *Faecalibacterium*, *Lachnospiraceae*, *Megamonas*, *Oscillospira*, *Sutterella* in Muscovy duck fed with increasing levels of HI larva meal<sup>1</sup>.



Note: HI: *Hermetia illucens*. <sup>1</sup>Four dietary treatments: HI0: control; HI3: 3% inclusion level of *Hermetia illucens*; HI6: 6% inclusion level of *Hermetia illucens*; HI9: 9% inclusion level of *Hermetia illucens*.

## 8.2 Exp 2

### 8.2.1 Growth performance

Table 23 depicts the growth performance results of the Exp 2. The LW, ADG, DFI and FCR were not affected by the dietary treatments ( $P > 0.05$ ).

**Table 23.** Effect on the growth performance of Muscovy ducks fed diet with live HI and TM live larvae.

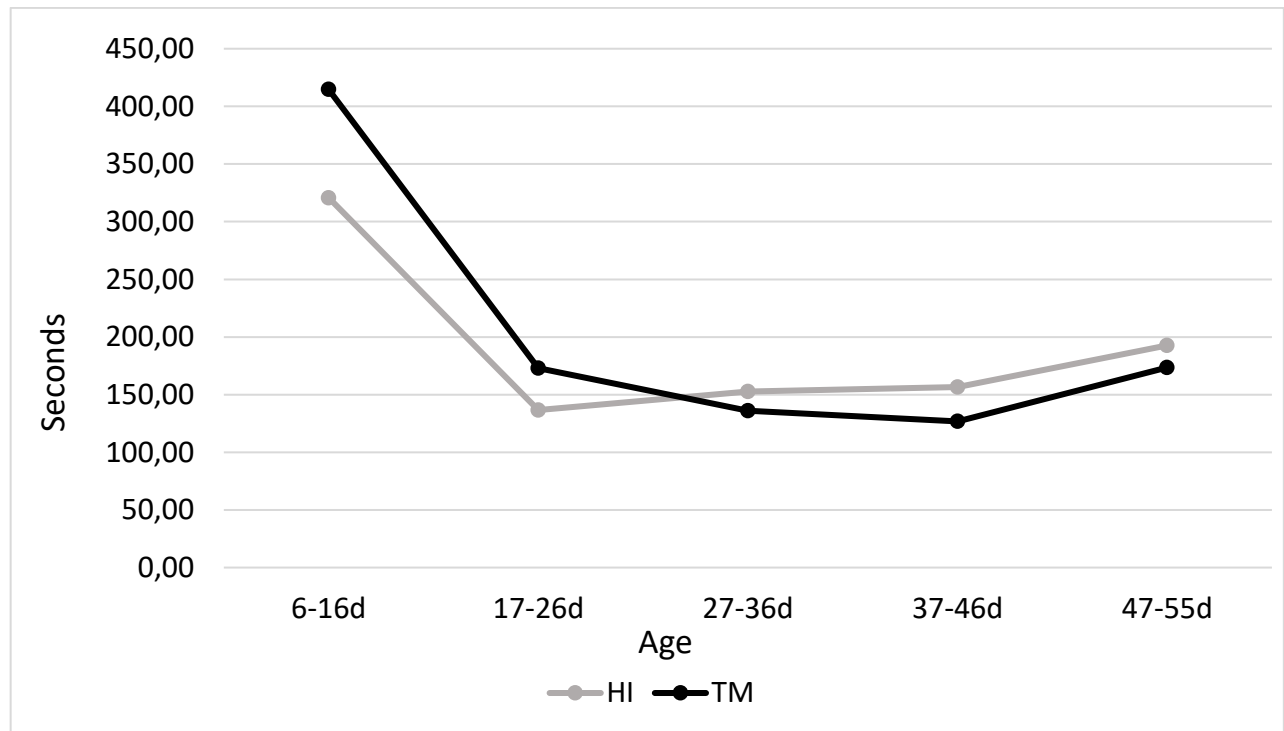
| Items     | Age     | Dietary treatments <sup>1</sup> |         |         | r.d.s | P-value |
|-----------|---------|---------------------------------|---------|---------|-------|---------|
|           |         | C                               | HI      | TM      |       |         |
| LW (g)    | 3 d     | 80.68                           | 80.19   | 78.69   | 3.33  | 0.588   |
|           | 31 d    | 1257.31                         | 1256.69 | 1230.14 | 48.29 | 0.567   |
|           | 55 d    | 2589.39                         | 2634.32 | 2560.69 | 83.30 | 0.322   |
| ADG (g)   | 3-31 d  | 42.02                           | 42.02   | 41.12   | 1.64  | 0.578   |
|           | 31-55 d | 55.50                           | 57.40   | 55.44   | 2.84  | 0.422   |
|           | 3-55 d  | 48.24                           | 49.12   | 47.73   | 1.58  | 0.324   |
| DFI (g)   | 3-31 d  | 81.18                           | 79.46   | 77.18   | 4.15  | 0.259   |
|           | 31-55 d | 176.22                          | 182.02  | 181.07  | 8.87  | 0.506   |
|           | 3-55 d  | 122.63                          | 124.39  | 124.32  | 6.34  | 0.876   |
| FCR (g/g) | 3-31 d  | 2.02                            | 1.98    | 1.93    | 0.06  | 0.055   |
|           | 31-55 d | 3.16                            | 3.15    | 3.13    | 0.19  | 0.960   |
|           | 3-55 d  | 2.58                            | 2.57    | 2.53    | 0.11  | 0.791   |

Note: C: control; HI: *Hermetia illucens*; TM: *Tenebrio molitor*; r.s.d: residual standard error; LW: live weight; ADG: average daily gain; DFI: daily feed intake; FCR: feed conversion ratio. <sup>1</sup>Three dietary treatments: C: control, fed commercial feed; HI: commercial feed + 5% HI larvae; TM: commercial feed + 5% TM larvae.

### 8.2.2 Behavioural observations

Larvae consumption times recorded in the Exp 2 are reported in Figure 12. The larvae consumption time was not different between the two larvae species provided to the ducks ( $P > 0.05$ ). However, the larvae consumption times were affected by the age of the birds, showing fastest time in larvae consumption between 27-36 days old ( $P < 0.05$ ).

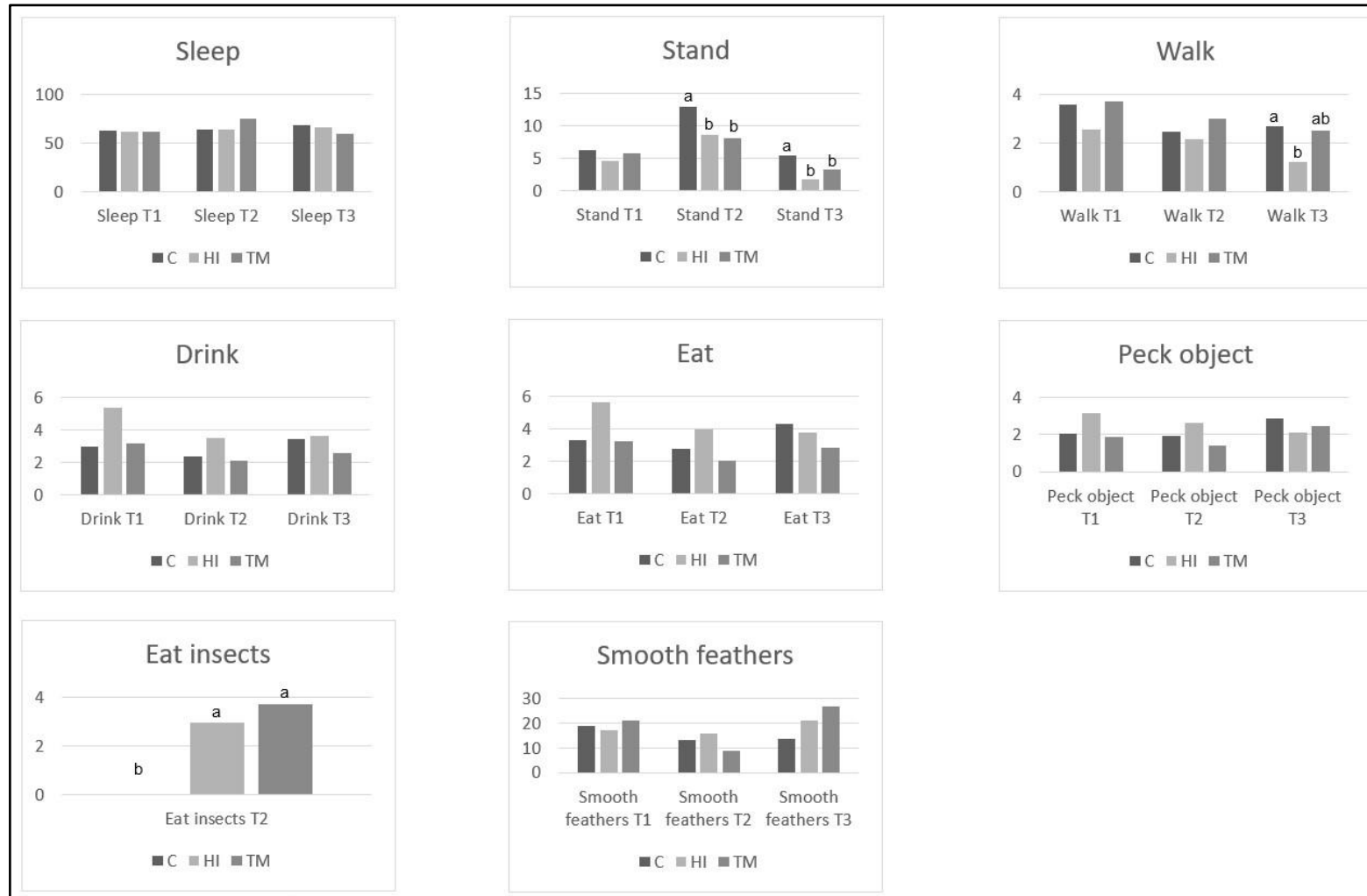
**Figure 12.** Larvae consumption times by the Muscovy ducks<sup>1</sup>.



Note: HI: *Hermetia illucens*; TM: *Tenebrio molitor*. <sup>1</sup>Three dietary treatments: C: control, fed commercial feed; HI: commercial feed + 5% HI larvae; TM: commercial feed + 5% TM larvae.

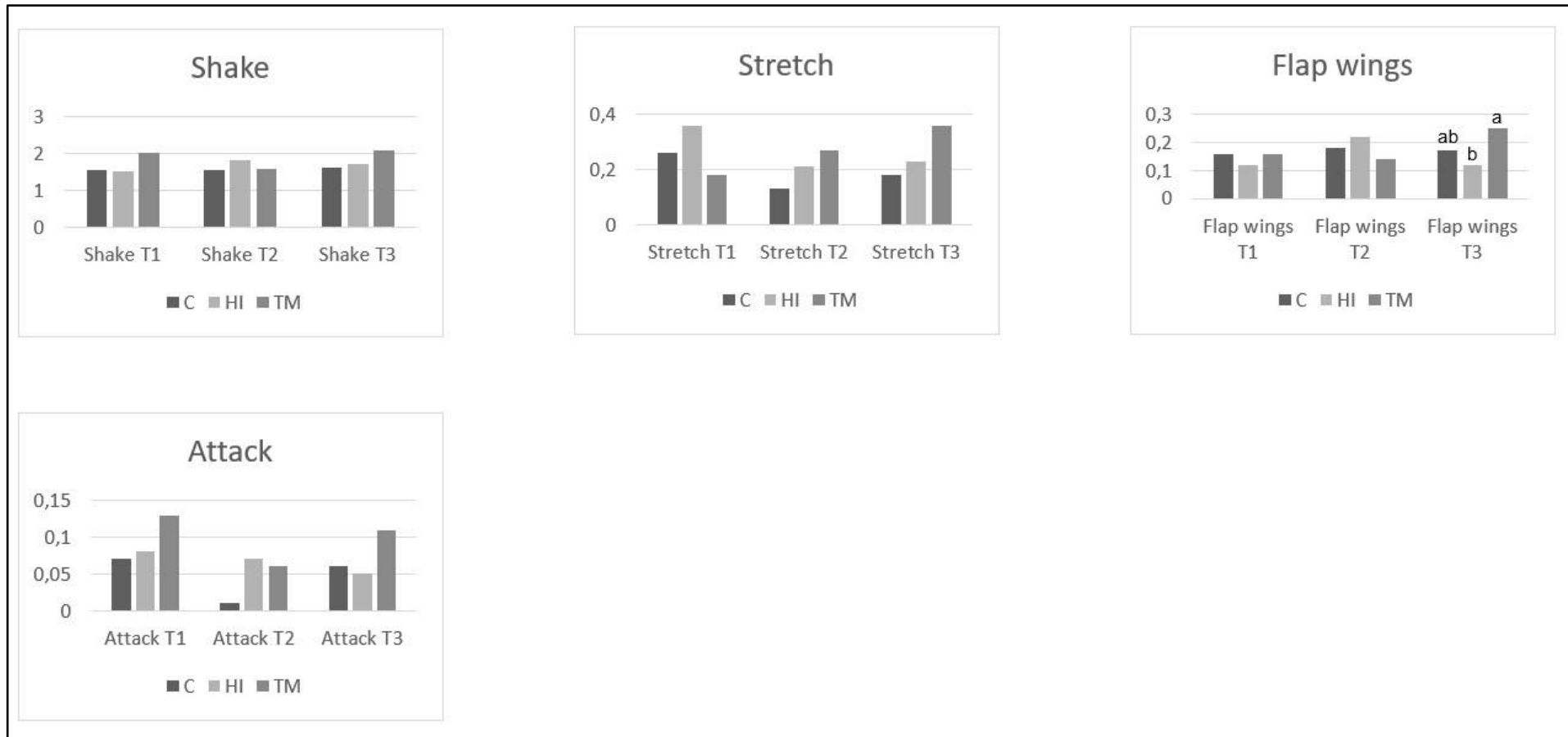
The behavioural observations of the state events are reported in Figure 13. During the time between 10.00-11.00 am (T2; hour of the larvae administration) and 11.00-12.00 am (T3; hour following the larvae provision), the birds of the control group showed higher time spent in stand position compared to the HI and TM groups [ $F(2,14) = 4.007$ ;  $P < 0.05$  and  $F(2,14) = 6.628$ ;  $P < 0.05$ , for T2 (10.00-11.00 am) and T3 (11.00-12.00 am), respectively]. Finally, during in the hour between 11.00-12.00 am (T3) the HI group showed lower time spent in walking activity compared to the control group [ $F(2,14) = 4.866$ ;  $P < 0.05$ ]. Figure 14 depicts the results obtained for the behavioural observation of the point events. During the hour between 11.00-12.00 am (T3) the TM group showed higher number of wings flapping compared to HI group [ $F(2,14) = 3.966$ ;  $P < 0.05$ ].

**Figure 13.** Behavioural observations of the state events of Muscovy ducks fed with HI and TM live larvae<sup>1</sup>.



Note: C: control; HI: *Hermetia illucens*; TM: *Tenebrio molitor*. <sup>1</sup>Three dietary treatments: C: control, fed commercial feed; HI: commercial feed + 5% HI larvae; TM: commercial feed + 5% TM larvae. Three observation times: T1: 09.00-10.00 am; T2: 10.00-11.00 am; T3: 11.00-12.00 am.

**Figure 14.** Behavioural observations of the point events of Muscovy ducks fed with HI and TM live larvae<sup>1</sup>.



Note: C: control; HI: *Hermetia illucens*; TM: *Tenebrio molitor*. <sup>1</sup>Three dietary treatments: C: control, fed commercial feed; HI: commercial feed + 5% HI larvae; TM: commercial feed + 5% TM larvae. Three observation times: T1: 09.00-10.00 am; T2: 10.00-11.00 am; T3: 11.00-12.00 am.

### 8.2.3 Blood analysis

The haematological and serum parameters of the Exp 2 are reported in Table 24. The haematological traits were not affected by the experimental treatments ( $P > 0.05$ ). However, the H/L ratio was affected by the dietary inclusion of live insects' larvae, resulting lower in the insects fed groups compared to the control ( $P < 0.05$ ). Serum protein, triglycerides and cholesterol levels were similar among groups, as well as the uric acid and creatinine levels ( $P > 0.05$ ). Similarly, AST, ALT, GGT were not different between groups ( $P > 0.05$ ).

**Table 24.** Effect of the dietary HI and TM live larvae inclusion on the haematological and serum parameters of Muscovy ducks.

| Items                                         | Dietary treatments <sup>1</sup> |                   |                   | r.s.d | P-value |
|-----------------------------------------------|---------------------------------|-------------------|-------------------|-------|---------|
|                                               | C                               | HI                | TM                |       |         |
| Erythrocytes, 10 <sup>6</sup> (cell/ $\mu$ L) | 2.85                            | 2.96              | 2.77              | 0.67  | 0.802   |
| Leukocytes, 10 <sup>3</sup> (cell/ $\mu$ L)   | 10.19                           | 9.61              | 8.36              | 3.41  | 0.418   |
| Heterophils (%)                               | 37.67                           | 38.00             | 36.08             | 5.94  | 0.627   |
| Lymphocytes (%)                               | 56.00                           | 58.91             | 59.08             | 5.75  | 0.346   |
| Monocytes (%)                                 | 2.33                            | 2.00              | 2.00              | 0.98  | 0.642   |
| Eosinophils (%)                               | 0.77                            | 1.42              | 1.67              | 1.02  | 0.070   |
| Basophils (%)                                 | 2.00                            | 1.17              | 1.08              | 1.18  | 0.107   |
| H/L                                           | 0.71 <sup>a</sup>               | 0.58 <sup>b</sup> | 0.57 <sup>b</sup> | 0.15  | 0.037   |
| Total Protein (g/dl)                          | 3.50                            | 3.12              | 3.08              | 0.91  | 0.520   |
| Triglycerides (mg/dl)                         | 54.68                           | 64.32             | 49.00             | 15.83 | 0.068   |
| Cholesterol (mg/dl)                           | 170.33                          | 152.62            | 130.12            | 61.43 | 0.282   |
| AST (U/l)                                     | 18.21                           | 17.00             | 16.67             | 5.81  | 0.802   |
| ALT (U/l)                                     | 7.91                            | 7.82              | 8.08              | 2.09  | 0.956   |
| GGT (U/l)                                     | 4.17                            | 4.50              | 4.50              | 1.32  | 0.789   |
| Uric acid (mg/dl)                             | 3.12                            | 2.94              | 2.69              | 0.91  | 0.538   |
| Creatinine (mg/dl)                            | 0.08                            | 0.08              | 0.07              | 0.02  | 0.810   |

Note: C: control; HI: *Hermetia illucens*; TM: *Tenebrio molitor*; r.s.d: residual standard error; H/L: heterophils to lymphocytes ratio; AST: aspartate-aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transferase.<sup>1</sup>Three dietary treatments: C: control, fed commercial feed; HI: commercial feed + 5% HI larvae; TM: commercial feed + 5% TM larvae.

### 8.2.4 Slaughtering performance

Table 25 depicts the results of the slaughtering performance of the Exp 2. The SW, carcass and organs yields were not affected by the dietary inclusion of live HI and TM larvae ( $P > 0.05$ ).

**Table 25.** Slaughtering performance of Muscovy duck fed live HI and TM larvae.

| Items                      | Dietary treatments <sup>1</sup> |         |         | r.s.d. | P-value |
|----------------------------|---------------------------------|---------|---------|--------|---------|
|                            | C                               | HI      | TM      |        |         |
| SW (g)                     | 2477.87                         | 2503.80 | 2510.97 | 66.53  | 0.452   |
| HC weight (g)              | 1857.66                         | 1868.88 | 1889.22 | 47.54  | 0.263   |
| HC yield (% SW)            | 75.00                           | 74.65   | 75.24   | 1.36   | 0.577   |
| CC weight (g)              | 1819.66                         | 1832.31 | 1849.25 | 47.43  | 0.317   |
| CC yield (% LW)            | 73.46                           | 73.19   | 73.65   | 1.33   | 0.708   |
| Breast yield (% CC weight) | 17.12                           | 17.04   | 16.70   | 1.50   | 0.777   |
| Thigh yield (% CC weight)  | 23.04                           | 22.77   | 22.74   | 1.23   | 0.817   |
| Spleen (% SW)              | 0.07                            | 0.07    | 0.07    | 0.01   | 0.785   |
| Liver (% SW)               | 1.79                            | 1.76    | 1.73    | 0.18   | 0.778   |
| Bursa of Fabricius (% SW)  | 0.15                            | 1.13    | 0.14    | 0.04   | 0.548   |
| Heart (% SW)               | 0.59                            | 0.59    | 0.63    | 0.06   | 0.204   |
| Abdominal fat (% SW)       | 1.43                            | 1.60    | 1.53    | 0.48   | 0.679   |

Note: HI: *Hermetia illucens*; TM: *Tenebrio molitor*; r.s.d: residual standard error; SW: slaughtering weight; HC: hot carcass; CC: chilled carcass. <sup>1</sup>Three dietary treatments: C: control, fed commercial feed; HI: commercial feed + 5% HI larvae; TM: commercial feed + 5% TM larvae.

### 8.2.5 Meat Quality Evaluation

The results related to pHu and L\*, a\*, b\* colour for BM and LM of the Exp 2 are reported in Table 26. No significant differences were observed for these parameters between experimental treatments (P > 0.05).

**Table 26.** pHu and colour of the breast meat (BM) and leg meat (LM) of Muscovy ducks fed live insects' larvae.

| Items |    | Dietary treatments <sup>1</sup> |       |       | r.s.d. | P-value |
|-------|----|---------------------------------|-------|-------|--------|---------|
|       |    | C                               | HI    | TM    |        |         |
| pHu   | BM | 5.75                            | 5.71  | 5.73  | 0.04   | 0.069   |
|       | LM | 5.84                            | 5.79  | 5.85  | 0.10   | 0.282   |
| L*    | BM | 47.59                           | 47.44 | 48.01 | 2.13   | 0.799   |
|       | LM | 49.38                           | 50.28 | 50.39 | 2.66   | 0.610   |
| a*    | BM | 11.11                           | 11.14 | 10.95 | 1.57   | 0.924   |
|       | LM | 7.80                            | 7.16  | 7.66  | 2.74   | 0.824   |
| b*    | BM | 6.24                            | 6.37  | 6.50  | 1.76   | 0.938   |
|       | LM | 6.48                            | 7.98  | 7.38  | 2.31   | 0.287   |

Note: HI: *Hermetia illucens*; TM: *Tenebrio molitor*; r.s.d: residual standard error; pHu: ultimate pH; L\*: lightness; a\*: redness; b\*: yellowness. <sup>1</sup>Three dietary treatments: C: control, fed commercial feed; HI: commercial feed + 5% HI larvae; TM: commercial feed + 5% TM larvae.

Table 27 depicts the breast water holding capacity results of the Muscovy duck fed with HI and TM live larvae. The results showed that the thawing and cooking losses were similar among groups, as well as the WBSF (P > 0.05).

**Table 27.** Breast water holding capacity of Muscovy duck fed with live insects' larvae.

| Items                       | Dietary treatments <sup>1</sup> |       |       | r.s.d. | P-value |
|-----------------------------|---------------------------------|-------|-------|--------|---------|
|                             | C                               | HI    | TM    |        |         |
| Thawing losses (%)          | 13.94                           | 12.34 | 12.62 | 1.77   | 0.057   |
| Cooking losses (%)          | 20.85                           | 21.47 | 21.43 | 2.26   | 0.762   |
| WBSF (kgf/cm <sup>2</sup> ) | 3.00                            | 3.07  | 3.02  | 0.31   | 0.872   |

Note: HI: *Hermetia illucens*; TM: *Tenebrio molitor*; r.s.d: residual standard error; WBSF: Warner-Bratzler shear force. <sup>1</sup>Three dietary treatments: C: control, fed commercial feed; HI: commercial feed + 5% HI larvae; TM: commercial feed + 5% TM larvae.

### 8.2.6 Histopathological and histomorphological investigations

The results of the organs histopathological investigation and intestinal histomorphological evaluations of the Exp 2 are reported in Tables 28 and 29, respectively. The experimental treatments did not affect the severity of the observed histopathological lesions in liver, thymus and Bursa of Fabricius ( $P > 0.05$ ). On the contrary, the spleen showed a greater white pulp hyperplasia in TM and HI groups compared to control ( $P < 0.05$ ) (Table 28).

**Table 28.** Histopathological scores of the Muscovy ducks fed HI and TM live larvae.

| Items              | Dietary treatments <sup>1</sup> |                   |                   | SEM   | P-value |
|--------------------|---------------------------------|-------------------|-------------------|-------|---------|
|                    | C                               | HI                | TM                |       |         |
| Spleen             | 0.03 <sup>a</sup>               | 0.35 <sup>b</sup> | 0.28 <sup>b</sup> | 0.051 | 0.025   |
| Liver              |                                 |                   |                   |       |         |
| Degeneration       | 0.57                            | 0.86              | 0.68              | 0.127 | 0.454   |
| Inflammation       | 0.00                            | 0.00              | 0.00              | 0.035 | 0.110   |
| Thymus             | 0.25                            | 0.11              | 0.21              | 0.044 | 0.438   |
| Bursa of Fabricius | 0.46                            | 0.32              | 0.57              | 0.063 | 0.306   |

Note: HI: *Hermetia illucens*; TM: *Tenebrio molitor*; SEM: standard error of the mean. <sup>1</sup>Three dietary treatments: C: control, fed commercial feed; HI: commercial feed + 5% HI larvae; TM: commercial feed + 5% TM larvae.

Regarding the histomorphological investigation, the Vh and the Cd values were affected by the considered intestinal segment, showing a proximo-distal decreasing gradient from duodenum to ileum ( $P < 0.001$ ). On the contrary, Vh/Cd resulted not affected ( $P > 0.05$ ). Furthermore, the dietary inclusion of HI and TM live larvae did not affect significantly the Vh, Cd and Vh/Cd in duodenum, jejunum and ileum ( $P > 0.05$ ; Table 29).

**Table 29.** Effects of diet, intestinal segment and interaction between diet and intestinal segment on the intestinal morphometric indices of the Muscovy ducks fed HI and TM live larvae.

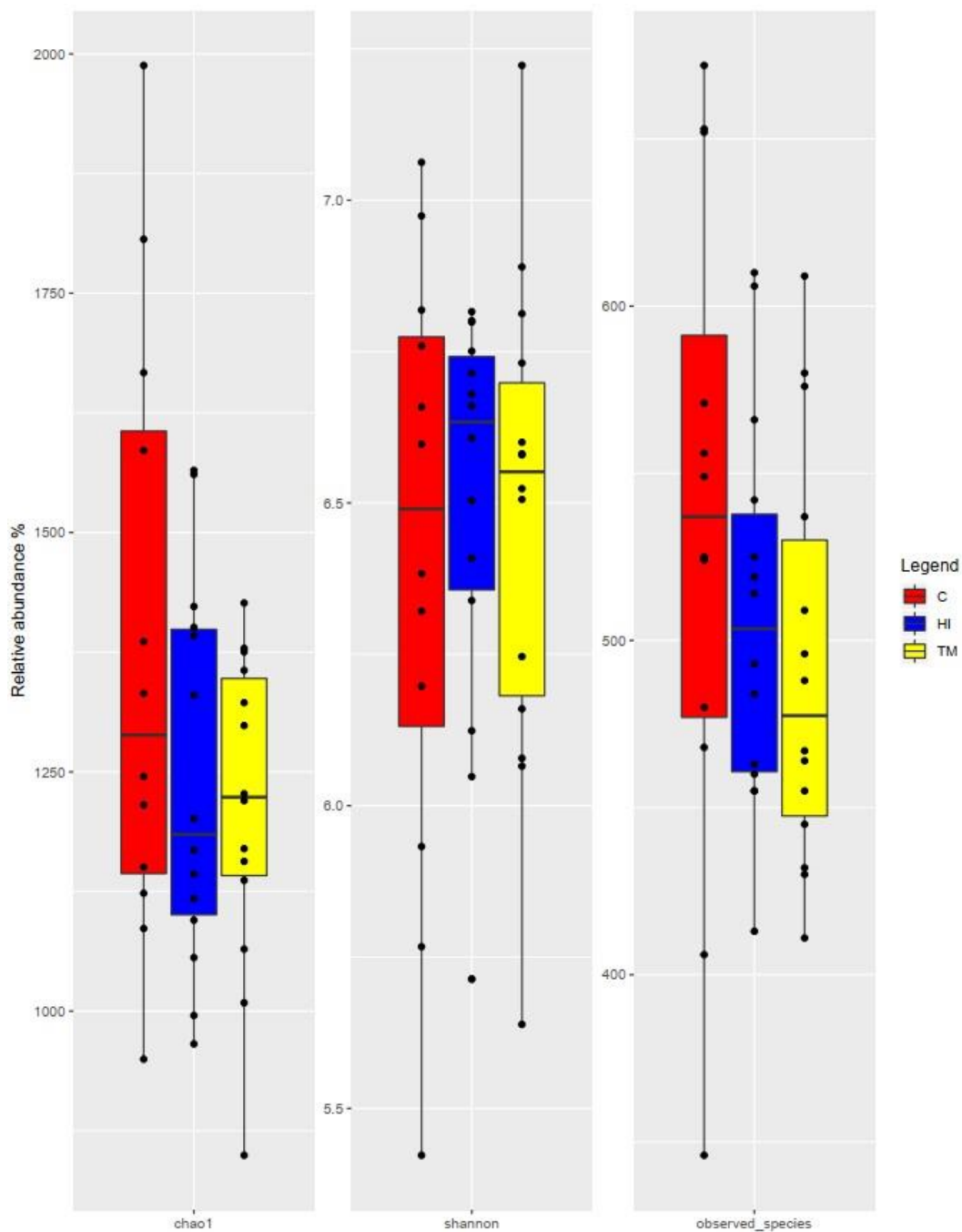
| Index   | Diets (D) <sup>1</sup> |       |       | Intestinal segments (IS) <sup>2</sup> |                   |                   | SEM   |       |       | P-value |        |
|---------|------------------------|-------|-------|---------------------------------------|-------------------|-------------------|-------|-------|-------|---------|--------|
|         | C                      | HI    | TM    | DU                                    | JE                | I                 | D     | IS    | D     | IS      | D x IS |
| Vh (mm) | 0.79                   | 0.78  | 0.84  | 1.03 <sup>a</sup>                     | 0.76 <sup>b</sup> | 0.62 <sup>c</sup> | 0.02  | 0.034 | 0.147 | <0.001  | 0.833  |
| Cd (mm) | 0.06                   | 0.07  | 0.07  | 0.08 <sup>a</sup>                     | 0.06 <sup>b</sup> | 0.05 <sup>c</sup> | 0.003 | 0.003 | 0.256 | <0.001  | 0.880  |
| Vh/Cd   | 12.17                  | 11.12 | 12.87 | 12.35                                 | 12.13             | 11.63             | 0.509 | 0.509 | 0.063 | 0.594   | 0.910  |

Note: HI: *Hermetia illucens*; TM: *Tenebrio molitor*; SEM: standard error of the mean; Vh: villus height; Cd: crypt depth; Vh/Cd: villus height-to-crypt depth ratio. <sup>1</sup>Three dietary treatments: C: control, fed commercial feed; HI: commercial feed + 5% HI larvae; TM: commercial feed + 5% TM larvae. <sup>2</sup>Three intestinal segments: DU = duodenum, JE = jejunum, IL = ileum.

### 8.2.7 Microbiota

At the end of the Exp 2, 36 caecal samples were obtained and sequenced. After sequencing and quality filtering 1420947 reads were used for downstream analysis with an average value of 33832 reads/sample. No significant differences in alpha diversity measures (Shannon and Chao1 indexes) were observed among the three experimental treatments ( $P > 0.05$ ; Figure 15).

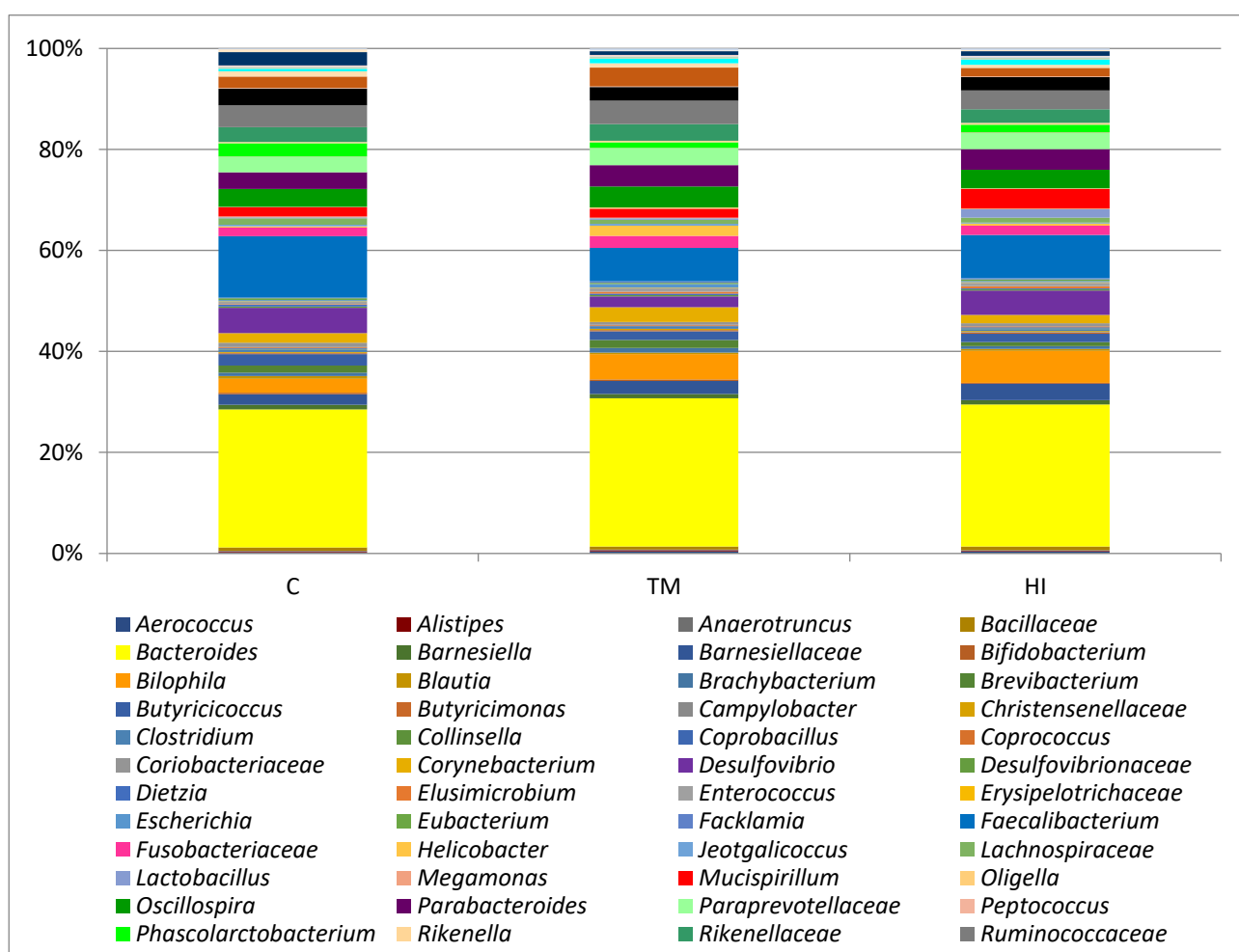
**Figure 15.** Alpha diversity measures (Chao1, Shannon and observed species indexes) of caecal microbiota of Muscovy duck fed live HI and TM larvae<sup>1</sup>.



Note: HI: *Hermetia illucens*; TM: *Tenebrio molitor*; <sup>1</sup>Three dietary treatments: C: control, fed commercial feed; HI: commercial feed + 5% HI larvae; TM: commercial feed + 5% TM larvae.

The relative abundances of bacterial taxa were examined to determine whether there were differences in microbiota composition between the experimental treatments (Figure 16). The microbiota composition showed a high predominance of *Bacteroides* (24.65, 26.66, and 24.93% of the relative abundance for C, TM, and HI respectively), *Faecalibacterium* (10.94, 6.01 and 7.62% for C, TM, and HI respectively), *Bilophila* (2.47, 4.56 and 5.62% for C, TM, HI respectively), *Ruminococcaceae* (3.91, 4.19, 3.32% for C, TM, HI respectively) and *Desulfovibrio* (4.52, 2, 4.25% for C, TM, and HI respectively).

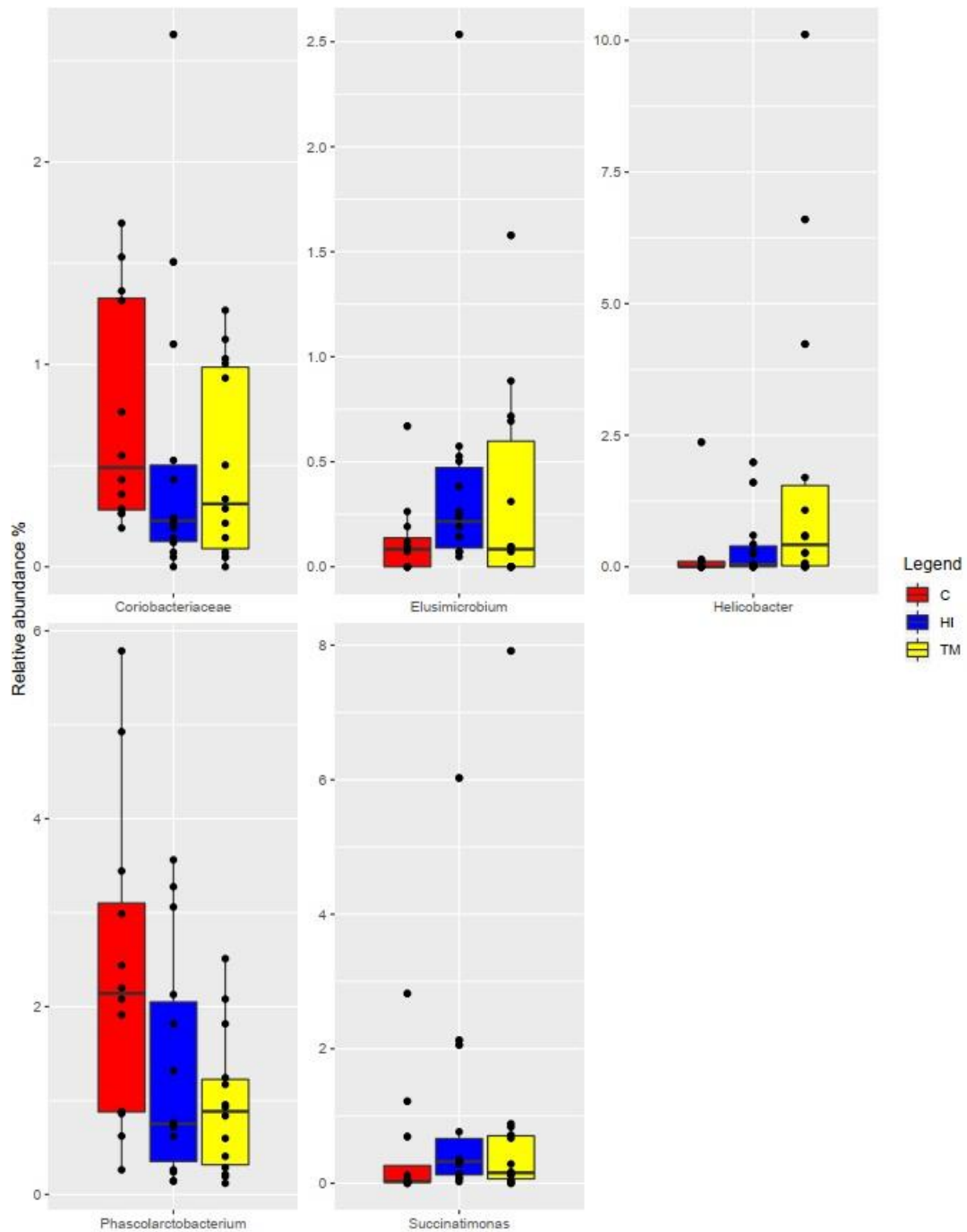
**Figure 16.** Relative abundance of the main bacterial families and genera in caecal samples of Muscovy ducks fed live HI and TM larvae<sup>1</sup>.



Note: HI: *Hermetia illucens*; TM: *Tenebrio molitor*; <sup>1</sup>Three dietary treatments: C: control, fed commercial feed; HI: commercial feed + 5% HI larvae; TM: commercial feed + 5% TM larvae.

However, the minor OTUs fraction was affected by the dietary treatments (Figure 17,  $P < 0.05$ ). In details, the C group was characterized by the presence of *Coriobacteriaceae* and *Phascolarctobacterium*, while the HI and TM groups showed a highest presence of *Helicobacter*, *Elusimicrobium*, and *Succinatimonas*.

**Figure 17.** Boxplots of *Coriobacteriaceae*, *Elusimicrobium*, *Helicobacter*, *Phascolarctobacterium*, *Succinatimonas* in Muscovy duck fed with live HI and TM larvae<sup>1</sup>.



Note: HI: *Hermetia illucens*; TM: *Tenebrio molitor*. <sup>1</sup>Three dietary treatments: C: control, fed commercial feed; HI: commercial feed + 5% HI larvae; TM: commercial feed + 5% TM larvae.

## 9. Discussion

Due to the partial similarity between the experimental design of two trials, the discussion will be addressed in a mixed way for the similar arguments. On the other hand, for specific evaluations performed in only one of the experimental trials, the discussion will be addressed in a specific way.

### 9.1 Growth performance

The final LW of the ducks of the two experimental trials resulted always higher compared to the rearing guide for their specific genotype (Canedins R71 L White and R61 Barred blue, respectively, Grimaud Freres Selection, France) at the same ages (Grimaud Freres, 2017). In Exp 1, despite the lower LW (38 days of age) and ADG (18-38 days of age) of the birds of HI6 group, their final LW and their overall ADG were similar between all the dietary treatments. On the other hand, the birds' growth performance in the Exp 2 resulted unaffected. Consistently with the obtained results, Cullere et al. (2016) reported unaffected final LW, BW gain, DFI, and FCR in broiler quails (*Coturnix coturnix japonica*) fed with HI meal at 10% and 15% inclusion levels in substitution of soybean meal. On the contrary, Loponte et al. (2017), evaluating dietary inclusion of TM and HI larva meals (in substitution of 25 and 50% of soybean meal) in Barbary partridges (*Alectoris barbara*) observed higher LW and better FCR in insects fed groups compared to the control. Regarding the dietary inclusion of other insects' species in broiler chickens, Biasato et al. (2017; 2018a) observed an improved LW and feed intake in male and female broiler chickens fed increasing dietary levels of TM larva meal (5, 10, 15% of inclusion).

For what the live larvae provision concern, few papers evaluate the effects of the dietary provision of live insects' larvae in poultry species. Veldkamp and van Niekerk (2019) evaluated the dietary inclusion of HI live larvae (10% of the expected DFI) in turkeys (*Meleagris gallopavo*) and their results were significantly different from the results obtained in the Exp 2 conducted in Muscovy ducks. Indeed, the daily provision of HI live larvae determined a higher DFI and BW gain, with a lower FCR than the control group. The observed differences in terms of growth performance could be probably related to the lower amount of larvae provided in Exp 2 compared to the work by Veldkamp and van Niekerk (2019) (5% vs. 10% of expected DFI). Indeed, HI and TM live larvae contain around 60-70% of water (Nowak et al., 2016; Star et al., 2020) and, for this reason, the amount of nutrients on a DM basis provided by the larvae was particularly low.

Generally, it is possible to assert that the dietary inclusion of partially defatted HI larva meal and, on the other hand, the dietary administration of HI and TM live larvae, did not have any negative effects on the ducks' growth performance. Moreover, further research is necessary in order to evaluate if a

higher amount of HI and TM live larvae provision in Muscovy duck could affect more significantly their growth performance.

## 9.2 Digestibility trial

The digestibility coefficients obtained in the Exp 1 were partially affected by the dietary inclusion of increasing levels of HI partially defatted meal. As was previously reported by Cullere et al. (2016) on broiler quails, the DM and OM total tract digestibility were not affected by the dietary inclusion of HI meal. Similarly, the inclusion up to 7.3% of HI meal did not negatively affect the apparent DM ileal digestibility in broiler chickens, while a reduction in apparent DM ileal digestibility was observed at higher inclusion level (14.6%) (Bovera et al., 2018). The reduced CP digestibility in the starter period for the groups with the higher dietary inclusion levels of HI larva meal (HI6 and HI9) could be related to the higher chitin content of these diets. It has been reported that chitin, the structural component of the insects' exoskeleton, could negatively influence the nutrient digestibility, and that of the CP in particular, since it results indigestible fibre for domestic poultry (De Marco et al., 2015). This result agrees with the results reported by Cutrignelli et al. (2018) and Bovera et al. (2018), that observed a reduced apparent ileal CP digestibility in laying hens and broiler chickens fed HI meals, respectively, compared to the control diet with soybean meal. However, in the Exp 1 the CP digestibility resulted similar in grower (18-38 days of age) and finisher (39-50 days of age) periods among groups. This result is particularly interesting, since it appears that there was an adaptation to the dietary chitin levels by the Muscovy ducks. Considering that insects are part of the natural diet of many wild birds' species and domestic poultry, it has been supposed that they could digest chitin better than other animals (Ravindran and Blair, 1993; Hossain and Blair, 2007). Recent studies conducted by Tabata et al. (2017; 2018) reported the presence of acid chitinase genes in bird's genome, and that the acid chitinase is mainly expressed in poultry's glandular stomach. The feeding behaviour of animals directly affects the acid chitinase mRNA in stomach tissue, resulting higher in omnivorous species compared to herbivorous and carnivorous ones (Tabata et al., 2018). Considering this hypothesis, it is possible that the higher dietary chitin levels of the HI6 and HI9 groups determined an increase in acid chitinase mRNA expression in the Muscovy ducks (in grower and finisher periods), leading to an improvement in CP digestibility compared to starter period. However, this speculation should be confirmed by future investigations. Moreover, a small increase in EE apparent digestibility was observed for the insect fed groups compared to the control (+1.97%, +1.28%, +1.60% on average for insects fed groups compared to the control, for starter, grower, and finisher periods, respectively). These highest EE digestibility are probably related to the higher overall amount of EE in the diets with HI larva meal

compared to the control diet. However, these slight effects on nutrients digestibility observed in Exp 1 did not affect the growth performance of the animals.

### **9.3 Behavioural observations**

One of the aims of Exp 2 was the evaluation of the behavioural modifications in Muscovy ducks by live HI and TM larvae provision. For what the larvae ingestion time concern, it is possible to observe an initial adaptation period to this new feed ingredient by the Muscovy ducks, requiring more time to the complete consumption of insect larvae at the beginning of the trial. Indeed, at the beginning of the trial the young Muscovy ducklings needed more time to get used to the larvae consumption, with about 7.4 min on average (445.5 sec) in the period between 1-10 days old. After the adaptation phase to the new ingredient, the ducks required less time to eat the larvae, reaching the faster time between 27-36 days old (2.10 min or 125.8 sec, on average). Instead, at the end of the trial, probably due to the animals' adaptation to the ingredient, the larvae ingestion time slightly increased, reaching around 3 min on average (183.1 sec). Few works evaluated the use of live insects' larvae in poultry species, and currently no studies have been conducted on ducks. The results obtained in the Exp 2, in terms of larvae ingestion time, are partially in agreement with Veldkamp and van Niekerk (2019), where the consumption of HI larvae (10% of the expected DFI) by turkeys was completed within 2 min after provision. However, in contrast with the results in Muscovy ducks, Veldkamp and van Niekerk (2019) reported no adaptation time for turkeys, already at the beginning of the trial.

The different insects' larvae species provided did not affect the ingestion times, as it can be also highlighted by the "eating insect" behaviour (Figure 13). Although the birds of the control group received the same manipulations of the insects fed groups by the operator (i.e., the plate presentation), a more fearful attitude was noticed by the control birds. Indeed, the higher time spent in standing position during 10.00-11.00 am and 11.00-12.00 am could be probably related to an "alert" behaviour of the birds of the control group. In ducks, the "alert" posture is performed standing, with the neck stretched and head erect (Johnsgard, 1965), and this behaviour was recorded mainly during operators' activity in the facility. On the contrary, the insect fed groups tended to approach the operator at larvae provision time and in general they appeared less fearful towards the operators. Moreover, also the walking activity resulted higher in the control group between 11.00-12.00 am, and this result is partially in agreement with the results obtained in turkeys, where the HI live larvae fed group showed less trotting/walking activity compared to the control (Veldkamp and van Niekerk, 2019). However, in contrast to Veldkamp and van Niekerk (2019), no differences were recorded in terms of foraging behaviours in the present trial. Similarly, also the frequency of injurious pecking was not affected by the dietary inclusion of live HI and TM larvae, while a reduction for this behaviour was recorded in

turkeys (Veldkamp and van Niekerk, 2019). The absence of effects on foraging and injurious pecking behaviours in Muscovy duck could be related to the small amount of larvae provided in the present trial than those provided to turkeys. Currently, to the best of the author's knowledge, this is the first experimental trial that evaluated the effects on ducks' behaviour by dietary provision of live insects' larvae, and further research are required to deeply investigate this possibility. It could be suggested that the provision of a higher amount of larvae could affect more significantly the birds' behaviours, resulting in more appreciable modifications on behavioural patterns.

## 9.4 Blood analysis

The blood parameters result of both the experimental trial provide information about the health status and the welfare of the animals. As previously reported for broiler chickens (Biasato et al., 2016; Biasato et al., 2018a; Dabbou et al., 2018b), laying hens (Marono et al., 2017; Bovera et al., 2018) and Barbary partridges (Loponte et al., 2017), the dietary inclusion of partially defatted HI larva meal, and also the dietary provision of live HI and TM larvae, did not have any detrimental effect on the haematological picture of the Muscovy duck. In the Exp 1 the H/L ratio was similar among the dietary treatments, highlighting the absence of any effect resulting from the dietary HI larva meal inclusion in Muscovy duck. On the contrary, in the Exp 2 the H/L ratio showed the lowest values in the insects fed groups compared to the control. The H/L ratio has been reported to be a sensitive haematological stress indicator in poultry, which increase in presence of environmental stressors or in reduced welfare conditions (Gross and Siegel, 1983; Pozzo et al., 2013). Despite the limited modifications in Muscovy ducks' behaviours observed in the Exp 2, the observed reduction of the H/L ratio results to be promising in terms of the improvement of animal welfare due to the dietary administration of live insect larvae.

Similarly, to the results obtained in laying hens (Marono et al., 2017; Bovera et al., 2018), reduced triglycerides and cholesterol levels were detected in the Exp 1. Different explanations could be associated to the blood reduction in triglycerides and cholesterol in the present work. Firstly, this reduction could be related to the chitin chelating effects of HI larva meal (Hossain and Blair 2007; Prajapati and Patel, 2010). Secondly, the microbiota composition of the birds of the Exp 1 showed a decrease in *Coriobacteriaceae* genus, following the increasing dietary HI larva meal levels. Some studies suggest that the *Coriobacteriaceae* could be associated to lipid and cholesterol metabolism (see chapter 9.8) (Martínez et al., 2013; Apajalahti and Vienola, 2016). However, in the Exp 2, probably due to the limited amount of nutrients provided by the live HI and TM larvae in terms of DM, the triglycerides and cholesterol levels were not different between groups.

In both the experimental trials AST, ALT, and GGT levels were not affected by the dietary treatments, and their value can be included in the normal ranges observed for poultry (Sakas, 2002; Schiavone et

al., 2018). The unaffected AST, ALT, and GGT levels indicate a good liver function of insects' fed Muscovy ducks. Moreover, in the Exp 1 a partially improved liver function was observed, with a reduction in ALP serum levels following the increasing dietary HI larva meal levels. Unchanged uric acid levels in both trials were observed. Moreover, a linear reduction in the creatinine levels in Muscovy ducks fed increasing HI larva meal in the Exp 1. These results agree with what was observed in broiler chickens fed increasing dietary levels of HI larva meal (Dabbou et al., 2018b). Similarly, Marono et al. (2017) reported unchanged AST, ALT, GGT, and ALP levels, as well as uric acid concentration in laying hens fed HI larva meal. Moreover, in Exp 1, the antioxidant enzymes and oxidative metabolites were also evaluated. In particular, the plasma oxidative metabolites were affected by the dietary HI larva meal inclusion, with a detected reduction in MDA and nitrotyrosine levels following the increasing dietary levels of HI meal. These results provide interesting information related to a reduced oxidative tissue damage (Ischiropoulos, 1998; Giannopoulou et al., 2002; Radi, 2013), that could be linked to the free radical scavenging effect of chitin. Despite the interesting result in terms of reduced oxidative metabolites in Exp 1, further researches are necessary to deeply investigate the chitin antioxidant effect.

In conclusion, in the Exp 1 the dietary HI larvae meal inclusion in Muscovy duck significantly affected the blood and serum parameters, with significantly biological effects linked to the new feed ingredient introduction. On the contrary, any direct nutritional effect was observed related to the dietary HI and TM live larvae inclusion in the Exp 2. From this point of view, the improved H/L ratio in insects fed groups of the Exp 2 is probably linked to a direct effect on animals' well-being due to the live larvae dietary inclusion, positively stimulated by the feeding behaviour. The haematological and serum results obtained in both the experimental trials highlight a preserved or even improved liver and renal functions, demonstrating the suitability of the dietary HI larva meal and, on the other hand, of HI and TM live larvae in Muscovy ducks.

## 9.5 Slaughtering performance

The slaughtering performance results of the two experimental trials are satisfying from a productive point of view. In general, the carcass yields were similar or even higher compared to the results obtained by Schiavone et al. (2004) and Kowalczyk et al. (2012) for Muscovy (HC yield = 61.8% SW) and Pekin ducks (HC yield = 66.4% SW), respectively. Despite in the Exp 1 a lower SW, HC and CC weights were observed for the group fed at 6% of dietary HI larva meal inclusion, the HC and CC yields of the HI6 group did not reflect this trend, resulting similar between groups. These results probably depended on the lower abdominal fat content of the slaughtered animals in the HI6 group compared to the other groups. Similarly, Cullere et al. (2016) reported unaffected carcass and breast yields in broiler quails fed increasing HI larva meal. Moreover, also Bovera et al. (2016) and Biasato et al. (2017) detected similar carcass, breast and thigh yields in broiler chickens fed TM larva meal. However, other studies conducted on broiler chickens showed increased carcass weights in HI fed groups compared to the control with soybean meal (Altmann et al., 2018; Schiavone et al., 2019).

## 9.6 Meat quality evaluation

The pHu values of BM and LM of Muscovy ducks were similar between the two trials, and in line with the values reported by Wawro et al. (2004) and Pingel et al. (2012) for Muscovy duck. The obtained results are in line with previous studies on Barbary partridges and broiler chickens fed dietary HI meal, where no differences were observed in pHu between dietary groups (Secci et al., 2018; Schiavone et al., 2019; Pieterse et al., 2018). However, the dietary inclusion of TM larva meal in broiler chickens revealed conflicting results in terms of pHu. Indeed, despite no differences were observed for this parameter in the study conducted by Secci et al. (2018) (inclusion of 12 and 22% of TM meal), the dietary inclusion of higher amount of TM meal (29.65%) determined higher pHu in insects fed group compared to the control (Bovera et al., 2016).

The L\* a\* b\* colour values observed for BM and LM in both the experiment resulted unaffected by the different dietary treatments. Similarly, unaffected colour values were reported in broiler chickens fed HI larva meal (Altmann et al., 2018; Pieterse et al., 2018; Secci et al., 2018; Schiavone et al., 2019), as well as for the dietary inclusion of TM meal (Secci et al., 2018).

In the Exp 1, the meat quality evaluation was deeply evaluated, since the dietary HI larva meal inclusion may affect the meat quality parameters more remarkably compared to the dietary HI and TM live larvae provision. Indeed, in the Exp 1 the meat proximate composition and oxidative status, FA profile, mineral and heavy metals concentrations were evaluated.

The BM and LM proximate compositions of the birds of the Exp 1 are in line with the values reported by Wawro et al. (2004) for Muscovy ducks. Moreover, the unaffected meat proximate composition is in line with previous studies on broiler quails, broiler chickens and Barbary partridge fed HI meal (Cullere et al., 2018; Pieterse et al. 2018; Secci et al., 2018). Similarly, the TBARS values (indicating the meat oxidative status) detected in the BM and LM were not affected by the dietary HI larva meal inclusion in Muscovy ducks of Exp 1, as previously reported by Cullere et al. (2018) in broiler quails fed HI larva meal (10 and 15% of inclusion). As for the meat mineral content concern, only the LM Cu content reported variations between groups, resulting higher in the HI9 group compared to the control. It could be speculated that this difference could be related to a difference in Cu bioavailability between vegetable and insect meals. Indeed, a low Cu bioavailability was determined in vegetable meals (Aoyagi and Baker, 1993; Leeson, 2009), while a higher Cu solubility has been reported in some insects' species (grasshoppers, crickets and mealworms) (Latunde-Dada et al., 2016).

According to previous studies, the most representative FA in Muscovy duck meat were myristic (C14:0), stearic (C18:0), oleic (C18:1 n-9) and linoleic (C18:2 n-6) acids (Aronal et al., 2012). However, due to the susceptibility of meat FA profile to the dietary FA profile in monogastric animals, changes in meat FA were observed both in BM and LM profile of the birds of the first trial (Schiavone et al, 2004; Schiavone et al, 2007; Schiavone et al., 2010). Indeed, accordingly to HI larva meal SFA profile, increasing lauric (C12:0) and myristic acids levels were detected both in Muscovy ducks' BM and LM. Because of these slight modifications, also the total SFA content in LM was affected by the dietary HI larva meal inclusion (+2.40% in HI9 group compared to the control), similarly to the results obtained by Cullere et al. (2018) in broiler quails fed HI larva meal. Moreover, meat  $\alpha$ -linolenic (C18:3 n-3) acid content was influenced by the dietary treatments. In particular,  $\alpha$ -linolenic acid showed opposite trends in BM and LM, with a decrease in BM for dietary HI meal inclusion levels, and an increase in LM (highest values observed for HI6 and HI9 groups). Another parameter that was affected by the dietary HI larva meal inclusion was the  $\Sigma n-6/\Sigma n-3$  ratios both in BM and LM, with their values (12.64 and 11.57 on average for BM and LM, respectively) that appeared higher compared to the 4:1 recommendation for human nutrition (Gómez Candela et al., 2011). However, since the FA composition of HI larvae could be modified by their rearing substrate, it could be possible to enrich larvae substrate with n-3 FA raw materials (i.e., for example, fish offal) in order to provide a richer n-3 insect meal to poultry (St-Hilaire et al., 2007). Finally, the AI (0.36 and 0.34 on average for BM and LM, respectively) and TI (0.94 and 0.84 on average for BM and LM, respectively) indexes were similar or even lower than the recommended values for poultry (0.55 and 0.95 for AI and TI, respectively) (Ulbricht and Southgate, 1991). As for the trace elements, As and heavy metal concentrations concern, their values resulted lower than the EU limits defined for foodstuffs (EC, 2006) in both BM and LM.

On the other hand, the thawing and cooking losses, as well as the WBSF were evaluated in Exp 2. No differences between experimental treatments were observed for the above-mentioned parameters. Our

results are partially in agreement with Cullere et al. (2016) that recorded similar breast cooking losses in broiler quails (fed up to 10% dietary HI larva meal) compared to the control, while higher cooking losses were detected at higher dietary HI larva meal level (15% of dietary inclusion of HI larva meal). Moreover, higher cooking losses were observed in broiler chickens breast fed 29% of inclusion of TM larva meal compared to the control with soybean meal (Bovera et al., 2016). The WBSF values obtained in the second trial are in line with the data reported by Omojola et al. (2007), that indicated a WBSF about 3.59 kg/cm<sup>2</sup> on average for Muscovy duck breast. The unaffected WBSF values observed in Exp 2 are in line with Bovera et al. (2016) on broiler chickens fed TM larva meal (29.65% of inclusion). In conclusion, the partially or even not affected pHu and color of birds of both the experimental trial, together with the meat proximate composition and oxidative status, mineral, and heavy metals concentrations evaluated in Exp 1, shows the suitability of the dietary inclusion of HI larva meal and HI and TM live larvae on Muscovy duck meat both from a nutritive and a safety point of view for the consumer. In Exp 1, only the FA profile was modified by the dietary HI larva meal inclusion, with the most interesting result represented by the reduction in  $\Sigma n-6/\Sigma n-3$  in the insect-fed group compared to the control. This result could represent a starting point for future investigations in order to evaluate the possibility of using HI meals obtained from n-3 rich substrates, with positive modifications in meat FA profile and, consequently, positive effects on consumers' health.

## **9.7 Histopathological and histomorphological investigations**

The dietary inclusion of HI larva meal, as well as HI and TM live larvae in Muscovy duck, did not affect their gut morphology. The dietary inclusion of these new feedstuffs in Muscovy duck did not negatively influenced the gut development, preserving the intestinal morphology and, consequently, without adversely affect the animals' performance. The obtained results in terms of gut morphology are in line with previous studies conducted in broilers (Uni et al., 1999; Murakami et al., 2007; Biasato et al., 2016; Biasato et al., 2017; Schiavone et al., 2017; Biasato et al., 2018a; Hall et al., 2018). Moreover, the dietary HI larva meal and HI and TM live larvae inclusions in Muscovy duck diet did not affect the severity of the organs' histopathological alterations in both Exp 1 and Exp 2.

Despite no difference in terms of absolute weight for the spleen of the birds in Exp 2 were observed, a greater with pulp hyperplasia was detected in Muscovy ducks fed HI and TM live larvae compared to the control. This result is partially in agreement with Bovera et al. (2016) that reported higher spleen weight in broiler chickens fed dietary TM larva meal. The greater spleen white pulp hyperplasia in insects' fed groups of the Exp 2 could suggest an increase in the immune system activity of these birds (Awadalla, 1998; Kusnadi and Djulardi, 2011). Despite this, the unaffected histopathological alterations of the other organs of both Exp 1 and Exp 2 are in line with Dabbou et al. (2018b) and Biasato et al.

(2016; 2017; 2018a), that did not report any significant organs alterations in broiler chickens fed dietary HI and TM larvae meal, respectively.

In conclusion, according to the present results, the dietary HI larva meal and HI and TM live larvae inclusion in Muscovy duck does not have any adverse effects on animal health.

## 9.8 Microbiota

The caecal microbiota composition of the ducks of both Exp 1 and Exp 2 were mainly characterized by *Bacteroides* and *Desulfovibrio* genus, as well as *Ruminococcaceae* family. Moreover, in the Exp 2, caecal microbiota was colonized also by *Faecalibacterium* genus and *Bilophila* spp. The obtained results are partially in agreement with Zhu et al. (2020), that reported *Bacteroides*, *Ruminococcaceae*, *Desulfovibrio*, and *Faecalibacterium* as some of the most representative genus and family bacteria in ducks' microbiota. Moreover, the present results are partially in line with what was observed in turkeys (Scupham et al., 2008) and geese (Xu et al., 2017).

Different findings in terms of alpha diversity measures were observed between the two experimental trials. Indeed, in Exp 1 an increase in alpha diversity was observed in Muscovy duck fed dietary HI larva meal at 3% of inclusion, while the lowest values were detected for HI6 and HI9 groups. This result is particularly interesting, since highest diversity levels provide the maintenance of intestinal microbiota stability in case of environmental stress and the resistance to invading pathogens colonization (Kuhn et al., 1993; Xue et al., 2015). On the contrary, in the Exp 2 no differences were observed in alpha diversity measures, confirming that the small amount of larvae provided has no negative effects on gut microbiota diversity.

However, a reduction in *Bacteroides* genus was observed in the HI9 group in the Exp 1. This result could be considered a potentially negative response related to the dietary HI larva meal inclusion. Indeed, *Bacteroides* genus is positively associated to the birds' intestinal health, having positive effects on animals' weight gain and growth performance (Chang et al., 2016; Biasato et al., 2018b). Moreover, it has been reported that the *Bacteroides* fermentation products could inhibit the *Clostridium perfringens* sporulation (Wrigley, 2004).

*Desulfovibrio* genus was detected in birds' caecum of both the experimental trails. The bacteria of this genus consume hydrogen for sulphate reduction, helping in the removal of the free hydrogen that is produced during anaerobic fermentations (Vasai, et al., 2014; Rychlik, 2020). On the other hand, *Ruminococcaceae* family represents major butyrate producers, with positive effects on enterocytes nourishment and on intestinal mucin production (Jung et al., 2015; Li et al., 2017). Moreover, butyrate is involved in differentiation and proliferation of mucosal cells and has an anti-inflammatory effect (Furusawa et al., 2013; Rinttila and Apajalahti, 2013).

In Exp 1, a reduction in *Coriobacteriaceae* family and *Parabacteroides* genus were observed at higher dietary HI larva meal inclusions. It has been reported that *Coriobacteriaceae* are connected to host lipid and cholesterol metabolism (Claus et al., 2011; Martínez et al., 2013). Indeed, higher faecal cholesterol concentrations have been related to a decrease of the relative *Coriobacteriaceae* abundance (Martínez et al., 2009). On the other hand, *Parabacteroides* (similarly to *Bacteroides* genus) produce bacteriocins, substances (proteins or peptides) that present a bactericidal effect (Nakano et al., 2006). The antagonistic effect of bacteriocins helps in the preservation of the resident microbiota and prevents the invasion and colonization of the exogenous bacteria, limiting the onset of infectious disease (Nakano et al., 2006).

Moreover, observing the OTUs fraction of the Exp 1, an increase in *Clostridium* genus was observed in HI9 group, while *Eubacterium* and *Faecalibacterium* genus showed the lower abundance compared to the other dietary treatments. *Clostridium* genus is one of the most representative members of poultry caecum microbiota (Pan and Yu, 2013; Rinttila and Apajalahti, 2013; Vasai, et al., 2014). Despite *Clostridium* genus is usually associated to pathogenic species (i.e., *Clostridium perfringens*), there are existing evidence that suggests that the majority of the species belonging to *Clostridium* genus are not pathogenic, including species that produce butyric acid, with positive effects on growth performance, structure of intestinal villus and with anti-inflammatory properties that helps in pathogen control (Rinttila and Apajalahti, 2013; Onrust et al., 2015). On the other hand, a reduction in *Eubacterium* and *Faecalibacterium* genus was observed in Exp 1. *Eubacterium* are one of the most important butyrate producers, with positive effects on enterocytes nourishment and on intestinal mucin production, as was previously reported for *Ruminococcaceae* family. Similarly, *Faecalibacterium* genus is generally associated to better FCR, high productivity and generally improved performance (Iqbal et al., 2020). The reduction of these two genera in Exp 1 could be considered a potentially negative response to the dietary HI larva meal inclusion.

In the Exp 2, *Helicobacter* genus resulted slightly higher in insects fed groups compared to the control. Since *Helicobacter* are usually negatively associated to birds' performance (Rychlik, 2020), this result could be considered as a negative effect related to the dietary inclusion of HI and TM live larvae in Muscovy duck. It has been reported that high levels of *Helicobacter* genus (and *Helicobacter pylori* in particular) could depress mucin synthesis, determining a worsening in terms of intestinal development (Deplancke and Gaskins, 2001; Rychlik, 2020). However, the gut structure and development of HI and TM live larvae fed groups were not negatively affected, as well as the birds' growth performance, showing that this increase in *Helicobacter* genus had no negative effects of birds' performance and welfare.

In conclusion, it is well known that nutrition, and therefore also the dietary insects' inclusion, directly affects the intestinal microbiota in poultry species (Biasato et al., 2018b; Biasato et al., 2019; Biasato et al., 2020). The effects of the dietary HI larva meal inclusion in Muscovy duck microbiota observed in

the first trial support this thesis. On the contrary, in Exp 2 this effect is not so much evident, probably due to the lower nutrients (in terms of DM) amount supplied by the live insects' larvae administration. Indeed, in Exp 2 the provision of live HI and TM larvae had greater effects on behavioural parameters compared to the strictly nutritional ones.

## 10. Conclusions

The objective of the present PhD research was the evaluation of the dietary inclusion of insect meal and live insect larvae in ducks. Two experimental trials were carried out: the first one consisted in four experimental diets with increasing levels of HI larva meal (0, 3, 6, 9% HI larva meal inclusion, respectively), while the second trial consisted in the daily provision of live HI and TM larvae (5% of expected average DFI). Different productive and welfare parameters were evaluated to assess the suitability of these new feed ingredients in ducks: growth and slaughtering performance, digestibility, behaviour, blood chemistry, meat quality parameters, organs histopathology and gut histomorphology assessment, and microbiota.

From a productive point of view, it could be asserted that the dietary inclusion both of HI larva meal and HI and TM live larvae in Muscovy duck did not have any detrimental effects on growth and slaughtering performance of the animals. The overall growth performance parameters were not negatively affected, resulting similar between groups. Moreover, satisfactory carcass yields in both the experimental trials were observed. Finally, the suitability of the dietary inclusion of HI larva meal in Muscovy duck is also confirmed by the good overall nutrient digestibility (Exp 1).

On the other hand, the behavioural patterns observed in Exp 2 were only slightly affected by the daily provision of live HI and TM larvae. As was previously reported, to the best of author knowledge this was the first trial aimed at evaluating the behavioural modification of Muscovy ducks fed live insects' larvae. Generally speaking, it was possible to observe an overall fear reduction in insect fed groups compared to the control, but probably the quantity of larvae provided was not adequate to markedly modify the birds' behaviour. Indeed, no effects were observed in terms of improved body care or, on the contrary, a reduction in attack occurrence. The provision of a higher amount of insect larvae could probably affect more significantly the ducks' behaviour, but further research are necessary in order to confirm this hypothesis.

The observed positive effects on haematological traits in both the experimental trial suggest that the dietary inclusion of both HI larva meal (Exp 1) and HI and TM live larvae (Exp 2) is suitable for Muscovy ducks. Indeed, significant differences were observed between the two experiments related to the type of feed ingredient used. In Exp 1 more significant effects were recorded, linked to the dietary inclusion of HI larva meal, while in Exp 2 an improvement in birds' welfare was detected.

Similarly, also the meat quality parameters were not affected by the dietary inclusion of HI larva meal (Exp 1) and HI and TM live larvae (Exp 2). As was previously observed in poultry, the meat FA profile is linked to the dietary FA profile, and the obtained results of Exp 1 confirm this thesis. However, the possibility to modify the insect FA profile could represent a good solution for a healthier meat (in terms of improved n-3 FA) for the consumers.

The unaffected organs' histology and gut morphology in both the experimental trial provide positive information about the dietary HI larva meal inclusion (Exp 1), as well as the dietary HI and TM live larvae supplementation (Exp 2), showing preserved intestinal and organs' functions.

In Exp 1 significant modifications in gut microbiota were observed, showing slightly negative effects at higher dietary HI larvae meal inclusion levels. However, in the light of what was observed for overall birds' performance, digestibility, and health in Exp 1, these little microbiota modifications are not harmful for the animals. On the other hand, no gut microbiota modification was observed in Exp 2, mainly related to the lower amount of nutrients provided by the live HI and TM larvae.

In conclusion, the present PhD project provides new information about insect dietary provision in Muscovy duck. The obtained results suggest that the dietary inclusion of HI larva meal and, on the other hand, the dietary provision of HI and TM live larvae are satisfactory for animal performance and well-being. However, further research is necessary in order to deeply investigate this opportunity, with the possibility to include different insects' species in ducks diet and, on the other hand, at different dietary inclusion levels.

Currently, the possibility to include these new feed ingredients in poultry nutrition is still a long way off, due to feed-ban restrictions in Europe and also due to their high cost that is not competitive to that of the currently used feed raw materials. However, this possibility must be taken in account with a view of improvement of animal production and welfare, together with a more respectful environment attention.

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# **Chapter 3**

## ***Research Products and Activities***

## 12. Research products

### 12.1 Articles

1. **Gariglio M.**, Dabbou S., Biasato I., Capucchio M.T., Colombino E., Hernández F., Madrid J., Martínez S., Gai F., Caimi C., Bellezza Oddon S., Meneguz M., Trocino A., Vincenzi R., Gasco L., Schiavone A. 2019. Nutritional effects of the dietary inclusion of partially defatted *Hermetia illucens* larva meal in Muscovy duck. J. Anim. Sci. Biotechnol. 10:37. doi: 10.1186/s40104-019-0344-7. (IF = 3.441; Q1).
2. **Gariglio M.**, Dabbou S., Crispo M., Biasato I., Gai F., Gasco L., Piacente F., Odetti P., Bergagna S., Plachà I., Valle E., Colombino E., Capucchio M.T., Schiavone A. 2019. Effects of the dietary inclusion of partially defatted black soldier fly (*Hermetia illucens*) meal on the blood chemistry and tissue (spleen, liver, thymus, and bursa of Fabricius) histology of Muscovy ducks (*Cairina moschata domestica*). Animals. 9: 307. doi:10.3390/ani9060307. (IF = 1.832; Q1).
3. Caimi C., Renna M., Lussiana C., Bonaldo A., **Gariglio M.**, Meneguz M., Dabbou S., Schiavone A., Gai F., Elia A.C., Prearo M., Gasco L. 2020. First insights on Black Soldier Fly (*Hermetia illucens* L.) larvae meal dietary administration in Siberian sturgeon (*Acipenser baerii* Brandt) juveniles. Aquac. 515: 734539. (IF = 3.224; Q1).
4. Castillo A., Schiavone A., Cappai M.G., Nery J., **Gariglio M.**, Sartore S., Franzoni A., Marzoni M. 2020. Performance of Slow-Growing Male Muscovy Ducks Exposed to Different Dietary Levels of Quebracho Tannin. Animals. 10(6):979. (IF = 2.323; Q1).

## 12.2 Abstracts and Posters

1. **Gariglio M.**, Dabbou S., Caimi C., Biasato I., Gai F., Capucchio M.T., Biasibetti E., Birolo M., Trocino A., Vincenzi R., Meneguz M., Gasco L., Schiavone A. Evaluation of carcass and meat traits of Muscovy duck fed with black soldier fly partially defatted meal. *Journal of Insects as Food and Feed*, Vol. 4:s38. The 2nd International Conference Insects to Feed the World (IFW 2018) Wuhan (China P.R.). May 15-18, 2018.
2. Meneguz M., Dama A., Caimi C., **Gariglio M.**, Schiavone A., Gai F., Gasco L. Preliminary result of high fibre material impact on growth performance of black soldier fly larvae (*Hermetia illucens*). *Journal of Insects as Food and Feed*, Vol. 4: s31. The 2nd International Conference 'Insects to Feed the World' (IFW 2018), Wuhan (China P.R.). May 15-18, 2018.
3. Gasco L., Schiavone A., Serra G., Anedda R., Trocino A., Caimi C., **Gariglio M.**, Malfatto V., Dabbou S., Devic E., Chatzifotis S., Meneguz M., Gai F. Apparent digestibility of insect protein meals for rainbow trout. *Journal of Insects as Food and Feed*, Vol. 4: s39. The 2nd International Conference 'Insects to Feed the World' (IFW 2018), Wuhan (China P.R.). May 15-18, 2018.
4. Caimi C., Varello K., Gai F., **Gariglio M.**, Meneguz M., Francese D. R., Dabbou S., Schiavone A., Prearo M., Bozzetto E., Gasco L. Histological evaluation in sturgeon's gut fed *Hermetia illucens* meal. *Journal of Insects as Food and Feed*, Vol. 4: s41. The 2nd International Conference 'Insects to Feed the World' (IFW 2018), Wuhan (China P.R.). May 15-18, 2018.
5. **Gariglio M.**, Dabbou S., Caimi C., Biasato I., Bellezza Oddon S., Meneguz M., Vincenzi R., Gai F., Biasibetti E., Capucchio M.T., Trocino A., Gasco L., Schiavone A. Effect of black soldier fly defatted meal on the growth performance and carcass yield of Muscovy duck: Preliminary results. *World's Poultry Science Journal*, Vol. 74: 63. 6th Mediterranean Poultry Summit (MPS 2018), Turin (Italy). June 18-20, 2018.
6. **Gariglio M.**, Dabbou S., Gasco L., Biasato I., Madrid Sanchez J., Martinez S., Gai F., Caimi C., Schiavone A. Digestibility of Muscovy duck fed with black soldier fly partially defatted meal. 22nd European Symposium on Poultry Nutrition (ESPN 2019), Gdansk (Poland), June 10-13, 2019.
7. Dabbou S., Lauwaerts I., Biasato I., Ferrocino I., Colombino E., Garcés Narro C., **Gariglio M.**, Gasco L., Capucchio M.T., Cocolin S., Schiavone A. Effect of modified insect oil on growth performance, carcass traits, gut morphology, histological features and fecal microbiota in broiler chicken diets. 22nd European Symposium on Poultry Nutrition (ESPN 2019), Gdansk (Poland), June 10-13, 2019.

8. Sartore S., Soglia D., Maione S., Dabbou S., **Gariglio M.**, Sacchi P., Rasero R., Mugnai C., Gasco L., Gai F., Schiavone A. Genetic diversity, productive and reproductive performance in Italian chicken breed Bianca di Saluzzo. 23rd ASPA Congress, Sorrento (Italy), June 11–14, 2019.
9. Soglia D., Sartore S., Maione S., **Gariglio M.**, Sacchi P., Rasero R., Mugnai C., Gasco L., Gai F., Schiavone A., Dabbou S. Genetic diversity, productive and reproductive performance in Italian chicken breed Bionda Piemontese. 23rd ASPA Congress, Sorrento (Italy), June 11–14, 2019.
10. **Gariglio M.**, Dabbou S., Gai F., Bellezza Oddon S., Trocino A., Guzmán A.P., Gasco L., Schiavone A. Meat proximate composition and fatty acids profile of Muscovy duck fed with partially defatted Black Soldier Fly meal. XVII European Symposium on the Quality of Eggs and Egg Products and XXIV European Symposium on the Quality of Poultry Meat (Egg & Meat 2019), Cesme (Turkey), June 23-26, 2019.
11. **Gariglio M.**, Dabbou S., Biasato I., Gai F., Colombino E., Capucchio M.T., Trocino A., Gasco L., Schiavone A. An overview of dietary effects of *Hermetia illucens* meal in Muscovy duck. XXIII Congress of the European Society of Veterinary and Comparative Nutrition (ESVCN 2019), Turin (Italy), September 18-20, 2019.

## 12.3 Congresses, Seminars and Awards

- ECM Event - Utilizzo degli insetti in alimentazione animale: stato dell'arte e prospettive [Grugliasco (Italy), 13/10/2017]
- VII Seminar de Nutricion de Animales de Compania [Murcia (Spain), 06/03/2018]
- 7th Workshop on Animal Production: from tradition to innovation [Beijing (China P.R.), 08/05/2018 – 22/05/2018]
- The 2nd International Conference Insects to Feed the World5B(IFW 2018) [Wuhan (China P.R.), 15/05/2018 – 18/05/2018]
- 6th Mediterranean Poultry Summit (MPS 2018) [Turin (Italy), 18/06/2018 – 20/06/2018]
- LVII National Congress Società Italiana Patologia Aviare (SIPA) [Forlì (Italy), 28/03/2019 – 29/03/2019]
- 22nd European Symposium on Poultry Nutrition (ESPN 2019) [Gdansk (Poland), 10/06/2019 – 13/06/2019]
- XVII European Symposium on the Quality of Eggs and Egg Products and XXIV European Symposium on the Quality of Poultry Meat (Egg & Meat 2019) [Cesme (Turkey), 23/06/2019 – 26/06/2019]
- XXIII Congress of the European Society of Veterinary and Comparative Nutrition (ESVCN 2019) [Turin (Italy), 18/09/2019 – 20/09/2019]
- Workshop - Impact of mycotoxins on food producing species: recent advances and future perspectives [Grugliasco (Italy), 25/10/2019]
- Winner of the "Prof. Giulia Giordani Award" as the BEST POSTER presentation in POULTRY NUTRITION [XXIII Congress of the European Society of Veterinary and Comparative Nutrition (ESVCN 2019), Turin (Italy), September 18-20, 2019].

## **13. Activities**

### **13.1 Stay Abroad**

- 23<sup>rd</sup> February 2018 – 24<sup>th</sup> March 2018: Department of Animal Production of the University of Murcia (Spain). Training activity in laboratory techniques under the supervision of Prof. Josefa Madrid Sánchez.


Due to the COVID-19 emergency, I was unable to complete the mandatory stays abroad period that I had planned in the months of April and May 2020 at the University of Wageningen (Netherlands), nor the month subsequently scheduled at the Department of Animal Production of the University of Murcia (Spain) in October 2020.

RESEARCH

Open Access



# Nutritional effects of the dietary inclusion of partially defatted *Hermetia illucens* larva meal in Muscovy duck

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## Abstract

**Background:** The present work is aimed at evaluating the effect of different inclusion levels of a partially defatted black soldier fly (*Hermetia illucens*, L.; HI) larva meal for ducks. A total of 192 female 3-day-old Muscovy ducklings (*Cairina moschata domestica*, Canedins R71 L White, Grimaud Freres Selection, France) were divided into 4 groups, assigned 4 different dietary treatments (6 replicates/treatment and 8 birds/replicate) and reared from 3 to 50 days of age. HI larva meal was included at increasing levels (0, 3%, 6% and 9%, HI0, HI3, HI6 and HI9, respectively) in isonitrogenous and isoenergetic diets formulated for 3 feeding phases: starter (3–17 days of age), grower (18–38 days of age) and finisher (39–50 days of age). The growth performance and apparent total tract digestibility were evaluated during the trial using titanium dioxide as an inert marker (0.3% of inclusion). At 51 days of age, two birds per pen were slaughtered and histomorphological investigations were performed.

**Results:** The live weight and average daily gain showed a quadratic response to increasing HI meal in the grower period (minimum corresponding to the HI6 group). No effects of dietary inclusion levels were observed for the daily feed intake or feed conversion ratio. The apparent dry matter and organic matter digestibility were not affected by the dietary treatment. A linear decrease was observed for the crude protein apparent digestibility in the starter period (minimum for the HI9 groups). The ether extract apparent digestibility increased linearly during the grower and finisher periods (minimum for the HI0 group). The morphometric indices were not influenced by the dietary treatments.

**Conclusions:** The inclusion of up to 9% of HI partially defatted larva meal in the diet of ducks did not cause any effect on growth performance, as well as the apparent digestibility. Moreover, dietary HI inclusion preserved the physiological intestinal development.

**Keywords:** Black soldier fly, Digestibility, Ducks, Histopathology, Insect, Performance

## Introduction

With an increase of 1.8% per year estimated until 2050, poultry production is one of the fastest growing sectors [1]. The production of duck meat has undergone a great expansion in recent years, passing from 2.23 million tonnes in 1996 to over 4.5 million tonnes in 2016 [2]. Duck

production plays an important role in Italy, with 3.27 thousand tonnes being produced in 2017 [3]. Duck farming has a great potential, since ducks are among the fastest producers of animal proteins and may contribute to the future nutritional needs of the growing world population [4].

The reduced availability of natural resources and the environmental impact of vegetable production require the research of alternative forms of protein for animal production. Considering the feeding habits of birds, including poultry (chicken, duck, turkey and geese), insects can represent a valuable alternative to the common

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protein sources, thanks to their nutritional characteristics [5]. The use of insects in feeds leads to advantages from both an environmental and nutritional point of view. Compared to conventional vegetable protein sources, insects rearing requires very little soil and water, and their low emission of greenhouse gases and ammonia implies an overall low environmental impact. A study conducted by an EU initiative PROteINSECT estimated that 1 ha of land can produce up to 150 tons of insect protein per year. Instead, 1 ha of land can produce less than 1 ton of soybean protein [6]. Moreover, insects can be reared on organic waste (such as fruit and vegetable) and can converting them into nutrients of high biological value, thanks to their high feed conversion efficiency [7, 8]. Researchers have recently been evaluating the use of insects in animal feeding, and they have highlighted that insects could be used as a partial or total substitute for the currently used protein sources. The most promising species are *Hermetia illucens* (HI) [9–12], *Musca domestica* [13, 14] and *Tenebrio molitor* (TM) [15–18]. Thanks to the favourable nutrient composition of HI, in terms of protein content (37–63%), amino acid profile, fat content (7–39%) and other macro- and micronutrients, it could be considered a valuable alternative to common protein sources, such as soybean meal (SBM) [19]. The effects of the inclusion of HI larva meal and oil on growth performance have been investigated in many studies [10–12, 20–22], with conflicting results being obtained. Only a few works are available regarding the digestibility of HI meal in poultry [9, 10, 22–24] and these show extremely variable results in terms of dry matter (DM), crude protein (CP) and ether extract (EE). In these studies, nutrient digestibility was frequently influenced, according to the authors' interpretation, by the chitin content of the diets, which can negatively affect digestibility. Despite the limited availability of studies, the evaluation of the gut histomorphometry represents a relevant research topic, since it could affect the growth performance and the digestibility of a diet [25], depending on the protein source and level of the diet [26, 27]. A recent study by Cutrignelli et al. [22] has found that the inclusion of HI larva meal in laying hen diets significantly affects the gut histomorphometry, resulting in a lower villi-to-crypt ratio than SBM group. The above-mentioned studies have shown a wide variability and more research is required to obtain a better understanding of the effect of the inclusion of HI in poultry diets.

To date, no research has investigated the possibility of including insect meal in duck diet. Therefore, the aim of the study was to evaluate the growth performance, digestibility and intestinal morphology of female broiler ducks (*Cairina moschata domestica*) fed diets including HI meal.

## Materials and methods

### Birds and husbandry

The present trial was performed at the poultry facility of the University of Turin (Italy). The experimental protocol (Prot. No. 380576, 4<sup>th</sup> December 2017) was approved by the Bioethical Committee of the University of Turin (Italy). The poultry house was 7 m wide × 50 m long × 7 m high, and was equipped with a waterproof floor and walls, completely covered by tiles and had an automatic ventilation system. A total of 192, 3-day-old, females Muscovy ducks (Canedins R71 L White, Grimaud Freres Selection, France) were housed in 24 pens and randomly allotted 4 dietary treatments, each group consisting of 6 pens as replicates with 8 birds per pen (average live weight (LW): 71.32 ± 2.95 g). Each pen was 1.20 m wide × 2.20 m long and was covered with rice hulls as litter. During the first 3 weeks, the animals were heated by infrared lamps. The lighting schedule was 23 h light : 1 h darkness until day 3 of the trial, and thereafter the dark period was gradually increased to 6 h and maintained constant until slaughtering. The environmental parameters were monitored daily during the whole period of the trial.

### Diets

Four dietary treatments were obtained in which increasing levels of a partially defatted HI larva meal (Hermetia Deutschland GmbH & Co KG, Baruth/Mark, Germany) were included. In order to assess the effect of HI meal inclusion in the diets, HI meal was included as a substitute to gluten meal, a commonly used raw material in commercial duck feeding, which nutritional value is comparable to HI meal.

The control group (HI0) was fed a diet without insect meal (9% inclusion of corn gluten meal); 3%, 6% and 9% of the gluten meal was substituted with HI larva meals in the HI3, HI6, and HI9 diets, respectively (Table 1).

The diets were isonitrogenous and isoenergetic and were formulated using the apparent metabolisable energy (AMEn) values for a partially defatted HI, which had been calculated by Schiavone et al. [23] and according to INRA for the other ingredients [28] both for broiler chickens. The essential amino acids requirements were calculated with matrix value for digestible amino acids according to Schiavone et al. [23] for HI meal and Pingel et al. [29] for the other ingredients, for chickens and ducks, respectively. Feed and water were provided *ad libitum* throughout the trial. A 3-phase feeding program was applied: a starter diet (days 3 to 17), a grower diet (days 18 to 38) and a finisher diet (days 39 to 51).

### Chemical analysis of the HI meal and experimental diets

The experimental diets were ground to pass through a 0.5-mm sieve and stored in airtight plastic containers.

**Table 1** Ingredients (g/kg as fed) and nutrient composition (%) of the experimental diets<sup>a</sup>

| Ingredients                             | Starter period (days 3 to 17) |       |       |       | Grower period (days 18 to 38) |       |       |       | Finisher period (days 39 to 50) |       |       |       |
|-----------------------------------------|-------------------------------|-------|-------|-------|-------------------------------|-------|-------|-------|---------------------------------|-------|-------|-------|
|                                         | HI0                           | HI3   | HI6   | HI9   | HI0                           | HI3   | HI6   | HI9   | HI0                             | HI3   | HI6   | HI9   |
| Corn meal                               | 600.0                         | 600.0 | 600.0 | 600.0 | 638.0                         | 638.0 | 638.0 | 638.0 | 670.0                           | 670.0 | 670.0 | 670.0 |
| Soybean meal                            | 212.0                         | 212.0 | 212.0 | 212.0 | 160.0                         | 160.0 | 160.0 | 160.0 | 100.0                           | 100.0 | 100.0 | 100.0 |
| HI larva meal                           | 0.0                           | 30.0  | 60.0  | 90.0  | 0.0                           | 30.0  | 60.0  | 90.0  | 0.0                             | 30.0  | 60.0  | 90.0  |
| Bran                                    | 42.5                          | 42.5  | 42.5  | 42.5  | 36.3                          | 36.3  | 36.3  | 36.3  | 66.2                            | 66.2  | 66.2  | 66.2  |
| Corn gluten meal                        | 90.0                          | 60.0  | 30.0  | 0.0   | 90.0                          | 60.0  | 30.0  | 0.0   | 90.0                            | 60.0  | 30.0  | 0.0   |
| Soybean oil                             | 16.5                          | 16.5  | 16.5  | 16.5  | 28.5                          | 28.5  | 28.5  | 28.5  | 34.5                            | 34.5  | 34.5  | 34.5  |
| Dicalcium phosphate                     | 10.0                          | 10.0  | 10.0  | 10.0  | 13.0                          | 13.0  | 13.0  | 13.0  | 4.0                             | 4.0   | 4.0   | 4.0   |
| Calcium carbonate                       | 8.0                           | 8.0   | 8.0   | 8.0   | 14.0                          | 14.0  | 14.0  | 14.0  | 17.4                            | 17.4  | 17.4  | 17.4  |
| Sodium chloride                         | 2.5                           | 2.5   | 2.5   | 2.5   | 2.5                           | 2.5   | 2.5   | 2.5   | 2.5                             | 2.5   | 2.5   | 2.5   |
| Sodium bicarbonate                      | 2.0                           | 2.0   | 2.0   | 2.0   | 2.0                           | 2.0   | 2.0   | 2.0   | 2.0                             | 2.0   | 2.0   | 2.0   |
| DL-methionine                           | 2.5                           | 2.5   | 2.6   | 2.8   | 1.7                           | 1.8   | 1.9   | 2.2   | 0.3                             | 0.4   | 0.5   | 0.8   |
| L-lysine                                | 3.9                           | 3.9   | 3.8   | 3.6   | 3.9                           | 3.8   | 3.7   | 3.4   | 3.0                             | 2.9   | 2.8   | 2.5   |
| Mineral-vitamin premix <sup>b</sup>     | 5.0                           | 5.0   | 5.0   | 5.0   | 5.0                           | 5.0   | 5.0   | 5.0   | 5.0                             | 5.0   | 5.0   | 5.0   |
| Choline chloride                        | 0.1                           | 0.1   | 0.1   | 0.1   | 0.1                           | 0.1   | 0.1   | 0.1   | 0.1                             | 0.1   | 0.1   | 0.1   |
| Optifos 250 bro                         | 1.0                           | 1.0   | 1.0   | 1.0   | 1.0                           | 1.0   | 1.0   | 1.0   | 1.0                             | 1.0   | 1.0   | 1.0   |
| Avizyme 1500x                           | 1.0                           | 1.0   | 1.0   | 1.0   | 1.0                           | 1.0   | 1.0   | 1.0   | 1.0                             | 1.0   | 1.0   | 1.0   |
| Titanium dioxide                        | 3.0                           | 3.0   | 3.0   | 3.0   | 3.0                           | 3.0   | 3.0   | 3.0   | 3.0                             | 3.0   | 3.0   | 3.0   |
| Total                                   | 1,000                         | 1,000 | 1,000 | 1,000 | 1,000                         | 1,000 | 1,000 | 1,000 | 1,000                           | 1,000 | 1,000 | 1,000 |
| AMEn <sup>c</sup> , kcal/kg             | 2,897                         | 2,892 | 2,888 | 2,884 | 2,994                         | 2,990 | 2,986 | 2,981 | 3,052                           | 3,048 | 3,044 | 3,040 |
| Nutrient composition <sup>d</sup>       |                               |       |       |       |                               |       |       |       |                                 |       |       |       |
| DM, %                                   | 89.3                          | 89.8  | 90.1  | 89.7  | 88.8                          | 89.2  | 89.1  | 89.1  | 88.7                            | 89.0  | 89.3  | 88.9  |
| CP, % DM                                | 25.1                          | 24.7  | 25.2  | 24.8  | 23.0                          | 22.5  | 22.5  | 22.4  | 20.2                            | 20.2  | 20.1  | 20.1  |
| EE, % DM                                | 4.7                           | 4.8   | 4.9   | 5.1   | 6.2                           | 6.2   | 6.4   | 6.6   | 7.3                             | 7.4   | 7.6   | 7.7   |
| NDF, % DM                               | 12.7                          | 12.3  | 12.7  | 12.4  | 12.9                          | 13.1  | 12.6  | 12.7  | 12.7                            | 13.1  | 12.7  | 12.9  |
| ADF, % DM                               | 3.4                           | 3.5   | 3.7   | 3.3   | 3.5                           | 3.5   | 3.7   | 3.5   | 3.4                             | 3.5   | 3.5   | 3.4   |
| Ash, % DM                               | 5.6                           | 6.0   | 5.6   | 5.8   | 7.8                           | 7.5   | 7.5   | 8.0   | 6.5                             | 6.4   | 6.9   | 6.7   |
| Fatty acid composition, % of total FAME |                               |       |       |       |                               |       |       |       |                                 |       |       |       |
| SFA                                     | 19.0                          | 20.7  | 23.9  | 26.9  | 17.7                          | 20.2  | 22.5  | 25.5  | 17.3                            | 19.4  | 22.2  | 23.7  |
| MUFA                                    | 27.6                          | 26.1  | 25.5  | 24.6  | 25.8                          | 24.9  | 24.2  | 23.5  | 24.9                            | 24.8  | 23.8  | 23.8  |
| PUFA                                    | 52.8                          | 52.6  | 49.9  | 47.9  | 55.9                          | 54.4  | 52.7  | 50.5  | 57.2                            | 55.3  | 53.5  | 51.8  |
| PUFA/SFA                                | 2.78                          | 2.54  | 2.08  | 1.78  | 3.15                          | 2.69  | 2.34  | 1.98  | 3.31                            | 2.85  | 2.40  | 2.18  |
| $\Sigma n-3$                            | 3.3                           | 3.4   | 3.4   | 3.3   | 4.1                           | 4.2   | 4.1   | 4.1   | 4.5                             | 4.5   | 5.3   | 5.0   |
| $\Sigma n-6$                            | 49.1                          | 48.9  | 46.1  | 44.3  | 51.6                          | 49.9  | 48.2  | 46.1  | 52.3                            | 50.4  | 47.9  | 46.4  |
| $\Sigma n-6/\Sigma n-3$                 | 14.88                         | 14.38 | 13.56 | 13.42 | 12.58                         | 11.88 | 11.76 | 11.24 | 11.62                           | 11.20 | 9.04  | 9.28  |

HI *Hermetia illucens*, AMEn apparent metabolisable energy, DM dry matter, CP crude protein, EE ether extract, NDF neutral detergent fiber, ADF acid detergent fiber, FAME fatty acid methyl esters, SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids

<sup>a</sup>Four dietary treatments: HI0 = control; HI3 = 3% inclusion level of *Hermetia illucens*; HI6 = 6% inclusion level of *Hermetia illucens*; HI9 = 9% inclusion level of *Hermetia illucens*

<sup>b</sup>Mineral-vitamin premix: vitamin A (retinyl acetate), 12,500 IU; vitamin D<sub>3</sub> (cholecalciferol), 3,500 IU; vitamin E (DL- $\alpha$ -tocopheryl acetate), 40 mg; vitamin K (menadione sodium bisulfite), 2.0 mg; biotin, 0.20 mg; thiamine, 2.0 mg; riboflavin, 6.0 mg; pantothenate, 15.21 mg; niacin, 40.0 mg; choline, 750.0 mg; pyridoxine, 4.0 mg; folic acid, 0.75 mg; vitamin B<sub>12</sub>, 0.03 mg; Mn, 70 mg; Zn, 62.15 mg; Fe, 50.0 mg; Cu, 7.0 mg; I, 0.25 mg; Se, 0.25 mg

<sup>c</sup>Calculated according to Schiavone et al. [23] for HI meal and INRA [28] for the other ingredients

<sup>d</sup>The chemical analyses were carried out on three replicates of each feed sample

HI larva meal and the experimental diets were analysed for DM (AOAC, method number #934.01), ash (AOAC, method number #942.05), CP (AOAC, method number #984.13), neutral detergent fiber (NDF; AOAC, method

number #2002.04) and acid detergent fiber (ADF; AOAC method number #973.18) [30]. The EE (AOAC, method number #2003.05) was determined according to International AOAC [31].

The chitin content of HI meal was determined according to Finke et al. [32] using ADF adjusted for its nitrogen content.

In order to perform the amino acids determination in HI meal, samples were prepared using a 22-h hydrolysis step in 6 mol/L HCl at 112 °C under a nitrogen atmosphere. Performic acid oxidation occurred prior to acid hydrolysis for methionine and cystine. The amino acids in hydrolysate was determined by means of HPLC after postcolumn derivatization, according to the procedure described by Madrid et al. [33]. Tryptophan was not determined. The lipid extraction and fatty acids profiling of the experimental diets [34–36] were carried out at the laboratory of the Department of Animal Medicine, Production and Health, University of Padua, Legnaro, Italy according to the method of Christie [37]. All the analysis were performed in triplicate (Table 1).

The chemical composition of the HI meal was the following: DM, 92.41 g/kg; CP, 56.71% DM; EE, 10.70% DM; ash, 16.38% DM; chitin, 6.43% DM; *DL*-methionine, 0.63% DM and *L*-lysine, 1.89% DM.

### Growth performance

Birds were individually labeled with a wing mark and weighed at their arrival. Mortality and clinical signs of illness were monitored daily throughout the trial. The live weight (LW) of the animals was recorded at an individual level at the beginning and at the end of each feeding phase (3, 17, 38 and 50 days of age), and the feeds were removed 2 h before the birds were weighed. The average daily gain (ADG) and average daily feed intake (DFI) were recorded at a pen level at the end of each growth period.

The feed conversion ratio (FCR) was calculated for each growth period and for the overall experimental period. All the measurements were made using electronic scales (Sartorius-Signum®, Bovenden, Germany).

### Digestibility trial

The digestibility trial was performed at the end of each feeding phase using titanium dioxide (TiO<sub>2</sub>, 0.3 g/kg) as an indigestible marker in each experimental diet (Table 1), in order to evaluate the apparent total tract digestibility coefficients (ATTDC). The Kaczmarek et al. [38] method was used to collect the excreta, with slight modifications, as reported by Dabbou et al. [39]. Briefly, all the birds were removed from each pen and housed in wire-mesh cages ( $n = 6$  replicates) for approximately 1 h/d for four consecutive days to collect fresh excreta samples.

After collection, the excreta samples, from which the feathers and litter had been removed, were immediately frozen at -20 °C. At the end of each collection period, the excreta were pooled, lyophilized, grounded and stored at 4 °C. All the analyses were carried out on two replicates for each sample. The ATTDC was evaluated for DM, CP,

EE and OM. The uric acid (UA) content in the excreta samples was determined spectrophotometrically (UNICAN UV-Vis Spectrometry, Helios Gamma, the United Kingdom) according to the Marquardt method [40]. The nitrogen contained in uric acid is the 33.33%. The CP amount in the excreta (CP *corrected*) was calculated using the excreta CP, corrected for UA as follows:

$$CP \text{ corrected} = (\text{total nitrogen} - \text{UA-nitrogen}) \times 6.25.$$

The TiO<sub>2</sub> content was measured on a UV spectrophotometer (UNICAN UV-vis Spectrometry, Helios Gamma, the United Kingdom) following the Myers et al. [41] method.

The ATTDC of the dietary nutrients was calculated using the following method [42]:

$$ATTDC X_{diet} = \left[ \frac{(\text{Total } X \text{ ingested} - \text{total } X \text{ excreted})}{\text{total } X \text{ ingested}} \right]$$

$$Digestibility = \left[ \frac{(\%X_{diet} / \%TiO_{diet}) - (\%X_{excreta} / \%TiO_{excreta})}{(\%X_{diet} / \%TiO_{diet})} \right]$$

where  $X$  represents DM, CP, EE or OM.

### Slaughtering procedures

At 50 days of age, the final LW of the birds was recorded individually and 12 ducks per diet (two birds per pen) were chosen on the basis of the LW pen average and identified by means of a shank ring. Then the feed was removed and, after 12 h of fasting (at 51 days of age), the selected animals were transferred to a commercial abattoir and slaughtered by electrical stunning and bleeding, according to the standard EU regulations. The plucked and eviscerated carcasses were obtained, the head, neck and feet were removed.

### Histomorphological investigations

Gut segments (approximately 5 cm in length, 12 animals per each group) of the duodenum, jejunum and ileum were sampled during slaughtering and flushed with 0.9% saline to remove all the contents. The collected intestine segments were the loop of the duodenum, the tract before Meckel's diverticulum (jejunum) and the tract before the ileocolic junction (ileum). The gut samples were fixed in a 10% buffered formalin solution, routinely embedded in paraffin wax blocks, sectioned at a 5-μm thickness, mounted on glass slides and stained with Haematoxylin & Eosin (HE) for morphometric analysis. The evaluated morphometric indices were as follows: villus height (Vh, from the tip of the villus to the crypt), crypt depth (Cd, from the base of the villus to the submucosa) and the villus height-to-crypt depth (Vh/Cd) ratio [27]. Morphometric analyses were performed on 10

well-oriented and intact villi and 10 crypts chosen from the duodenum, jejunum and ileum [26].

### Statistical analysis

The statistical analyses were performed using the SPSS software package (version 21 for Windows, SPSS Inc., Chicago, IL, USA). The mortality rate was analysed by means of a Chi-square test, using the HI0 group as the reference. Shapiro-Wilk's test established normality or non-normality of distribution. The assumption of equal variances was assessed by means of Levene's homogeneity of variance test. The experimental unit was the pen for growth performance and digestibility, while the duck was used for the intestinal morphology. The collected data were tested by means of one-way ANOVA. Polynomial contrasts were used to test the linear and quadratic responses to increases in the HI inclusion level in the diet. Intestinal morphometric indices were analysed by fitting a general linear mixed model (GLMM). GLMM allowed the morphometric indices (Vh, Cd and Vh/Cd, separately) to depend on three fixed factors (diet, intestinal segment and interaction between diet and intestinal segment). Animal was included as a random effect to account for repeated measurements in the same duck. The interactions between the levels of the fixed factors were evaluated by means of pairwise comparisons.

Differences among treatments were considered statistically significant when the  $P$  values  $\leq 0.05$ .

## Results

### Growth performance

The cumulative mortality rates of the HI0 (4.16%), HI3 (2.08%), HI6 (2.08%) and HI9 (2.08%) groups were not influenced by the dietary treatments ( $P > 0.05$ ). The growth performances of the broiler ducks are summarized in Table 2. Overall LW was not influenced by the dietary treatments ( $P > 0.05$ ), except at 38 days of age, when a quadratic response was observed in LW for increasing HI meal levels with a minimum being observed for the HI6 group ( $P < 0.05$ ).

ADG was not affected by the dietary treatments ( $P > 0.05$ ), with the exception of the HI6 group in the second period (18–38 days of age), where the ADG showed a quadratic response ( $P < 0.05$ ). DFI and FCR were not affected by the dietary treatment nor in the different feeding phases or over the whole experimental trial ( $P > 0.05$ ).

### Digestibility trial

The apparent digestibility coefficients are reported in Table 3. DM digestibility was not affected by the dietary treatment throughout the trial, as well as the OM ( $P > 0.05$ ). In the starter period (3–17 days of age) the CP digestibility decreased linearly with a minimum corresponding to the HI9 groups ( $P < 0.05$ ) (–4.11% compared to HI0, respectively), whereas the EE digestibility decreased linearly with the inclusion of HI in the diets ( $P < 0.05$ ).

**Table 2** Effect of the dietary HI larva meal inclusion on the growth performance of female ducks ( $n = 6$ )

| Items    | Age     | Dietary treatments <sup>a</sup> |         |         |         | SEM   | P-value |           |
|----------|---------|---------------------------------|---------|---------|---------|-------|---------|-----------|
|          |         | HI0                             | HI3     | HI6     | HI9     |       | Linear  | Quadratic |
| LW, g    | 3 d     | 70.70                           | 70.41   | 72.65   | 71.51   | 0.60  | 0.405   | 0.733     |
|          | 17 d    | 575.44                          | 567.31  | 572.56  | 575.74  | 4.76  | 0.893   | 0.582     |
|          | 38 d    | 1906.79                         | 1861.96 | 1797.10 | 1900.12 | 14.18 | 0.426   | 0.005     |
|          | 50 d    | 2540.57                         | 2511.14 | 2456.14 | 2554.84 | 20.13 | 0.946   | 0.123     |
| ADG, g/d | 3–17 d  | 36.05                           | 35.49   | 35.71   | 36.02   | 0.32  | 0.974   | 0.529     |
|          | 18–38 d | 63.40                           | 61.65   | 58.31   | 63.07   | 0.69  | 0.417   | 0.011     |
|          | 39–50 d | 52.81                           | 54.10   | 54.92   | 54.56   | 1.14  | 0.582   | 0.738     |
|          | 3–50 d  | 52.55                           | 51.93   | 50.71   | 52.84   | 0.43  | 0.926   | 0.125     |
| DFI, g/d | 3–17 d  | 53.69                           | 53.45   | 52.16   | 51.85   | 0.59  | 0.226   | 0.979     |
|          | 18–38 d | 142.03                          | 139.06  | 136.99  | 139.92  | 1.28  | 0.481   | 0.273     |
|          | 39–50 d | 167.48                          | 170.82  | 160.32  | 171.80  | 2.83  | 0.924   | 0.485     |
|          | 3–50 d  | 120.58                          | 121.32  | 117.58  | 121.48  | 1.20  | 0.927   | 0.530     |
| FCR, g/g | 3–17 d  | 1.49                            | 1.51    | 1.46    | 1.44    | 0.01  | 0.099   | 0.489     |
|          | 18–38 d | 2.24                            | 2.26    | 2.35    | 2.22    | 0.03  | 0.913   | 0.159     |
|          | 39–50 d | 3.17                            | 3.17    | 2.93    | 3.18    | 0.051 | 0.639   | 0.220     |
|          | 3–50 d  | 2.29                            | 2.34    | 2.32    | 2.30    | 0.019 | 0.925   | 0.406     |

HI *Hermetia illucens*, SEM standard error of the mean, LW live weight, ADG average daily gain, DFI daily feed intake, FCR feed conversion ratio

<sup>a</sup>Four dietary treatments: HI0 = control; HI3 = 3% inclusion level of *Hermetia illucens*; HI6 = 6% inclusion level of *Hermetia illucens*; HI9 = 9% inclusion level of *Hermetia illucens*

In the periods from 18 to 38 days of age and from 39 to 50 days of age, the EE digestibility showed a linear increase, with a maximum corresponding to the HI9 group ( $P < 0.001$ ) (+ 1.94% and + 3.05% compared to HI0 in the grower and finisher periods, respectively). However, CP digestibility was not affected by the dietary treatments in the grower and finisher periods ( $P > 0.05$ ).

### Histomorphological investigations

The effects of the diet, gut segment and interaction between the diet and gut segment on the gut morphometric indices of the ducks are summarized in Table 4. The intestinal segment significantly affected Vh, Cd and Vh/Cd ( $P < 0.001$ ). On the other hand, no influence of diet or interaction between the diet and intestinal segment ( $P > 0.05$ ) were observed on the morphometric indices. The duodenum showed higher Vh and Cd values ( $P < 0.05$  and  $P < 0.01$ , respectively) than the ileum, and the morphometric indices were also greater ( $P < 0.05$  and  $P < 0.01$ , respectively) in the jejunum than in the ileum. The duodenum also showed a greater Vh/Cd ( $P < 0.05$ ) than the other gut segments.

## Discussion

### Growth performance

Currently, no literature is available regarding the use of insect meals in duck feeding. For this reason, all the comparisons with literature data referred to other poultry species fed with HI meals and other insect meals.

The final LW of the birds was in line with the weight reported by Pingel et al. [29]. The results showed that HI meal could be a valuable alternative to corn gluten meal, and HI meal can be included in duck diets by as

much as 9% without any negative effects on the final LW, ADG, DFI and FCR of the animals. Despite the lower LW and ADG of HI6 birds in the grower period (18–38 days of age), DFI and FCR were not influenced by the dietary treatment, and the final LW of the HI6 group was in line with the weight of the other treatments. Similarly, Cullere et al. [10] did not observe any differences in the final LW of broiler quails (*Coturnix coturnix japonica*) fed two different diets at 10% and 15% of inclusion levels of HI meal (in substitution of SBM protein and oil). Our results also agree with what Bovera et al. [24] previously reported for laying hens fed 25% and 50% HI in substitution of SBM (73 g/kg and 146 g/kg of inclusion, respectively), thus showing that LW and DFI were not influenced by the dietary treatments. The inclusion of up to 10% of HI larva meal in the diet of broiler chickens influenced their final LW and also improved the DFI of chicks in the starter period [12]. As far as other insect species with potential interest as feeds are concerned, Adenjii [13] did not observe any dietary effects on the performance of broiler chickens when groundnut cake was substituted with housefly maggot (*M. domestica*) meal. Biasato et al. [16], Ramos-Elorduy et al. [43] and Bovera et al. [44] also reported that the inclusion of TM meal in broiler chicken diets (from 5% to 15% of inclusion) did not affect the final LW and DFI of the birds. On the other hand, the replacement of SBM with 25% and 50% of HI meal (100 and 190 g/kg of inclusion, respectively) and 25% TM meal (120 g/kg of inclusion) in Barbary partridge (*Alectoris barbara*) resulted in a higher LW than the control [45]. Finally, the results reported by Khan et al. [46], pertaining to broiler chicks fed with silkworm (*Bombyx mori*), housefly maggot and TM in substitution of SBM

**Table 3** Effect of the dietary inclusion of HI larva meal on the nutrients apparent digestibility of Muscovy ducks ( $n = 6$ )

| Age     | Apparent digestibility | Dietary treatments <sup>a</sup> |       |       |       | SEM   | P-value |           |
|---------|------------------------|---------------------------------|-------|-------|-------|-------|---------|-----------|
|         |                        | HI0                             | HI3   | HI6   | HI9   |       | Linear  | Quadratic |
| 3–17 d  | DM                     | 0.960                           | 0.960 | 0.960 | 0.953 | 0.002 | 0.174   | 0.315     |
|         | CP                     | 0.852                           | 0.872 | 0.828 | 0.817 | 0.007 | 0.010   | 0.195     |
|         | EE                     | 0.945                           | 0.967 | 0.963 | 0.962 | 0.002 | 0.003   | 0.085     |
|         | OM                     | 0.963                           | 0.963 | 0.966 | 0.962 | 0.001 | 0.853   | 0.453     |
| 18–38 d | DM                     | 0.953                           | 0.956 | 0.962 | 0.960 | 0.002 | 0.086   | 0.511     |
|         | CP                     | 0.800                           | 0.802 | 0.802 | 0.828 | 0.005 | 0.085   | 0.269     |
|         | EE                     | 0.958                           | 0.966 | 0.968 | 0.977 | 0.002 | < 0.001 | 0.891     |
|         | OM                     | 0.958                           | 0.964 | 0.967 | 0.963 | 0.001 | 0.215   | 0.168     |
| 39–50 d | DM                     | 0.943                           | 0.948 | 0.952 | 0.953 | 0.002 | 0.099   | 0.703     |
|         | CP                     | 0.733                           | 0.682 | 0.715 | 0.718 | 0.012 | 0.913   | 0.259     |
|         | EE                     | 0.953                           | 0.958 | 0.965 | 0.983 | 0.003 | < 0.001 | 0.092     |
|         | OM                     | 0.950                           | 0.953 | 0.958 | 0.958 | 0.002 | 0.072   | 0.642     |

HI *Hermetia illucens*, SEM standard error of the mean, DM dry matter, CP crude protein, EE ether extract

<sup>a</sup>Four dietary treatments: HI0 = control; HI3 = 3% inclusion level of *Hermetia illucens*; HI6 = 6% inclusion level of *Hermetia illucens*; HI9 = 9% inclusion level of *Hermetia illucens*

**Table 4** Intestinal morphometric indices in the ducks in relation to diet and intestinal segment ( $n = 12$ , end of the trial)

| Index  | Diet (D) <sup>d</sup> |       |       |       | Intestinal segment (IS) <sup>e</sup> |                   |                   | SEM  |      | P-value |         |        |
|--------|-----------------------|-------|-------|-------|--------------------------------------|-------------------|-------------------|------|------|---------|---------|--------|
|        | HI0                   | HI3   | HI6   | HI9   | DU                                   | JE                | IL                | D    | IS   | D       | IS      | D × IS |
| Vh, mm | 1.55                  | 1.51  | 1.52  | 1.63  | 2.12 <sup>a</sup>                    | 1.41 <sup>b</sup> | 1.14 <sup>c</sup> | 0.06 | 0.06 | 0.442   | < 0.001 | 0.508  |
| Cd, mm | 0.16                  | 0.14  | 0.15  | 0.15  | 0.18 <sup>a</sup>                    | 0.15 <sup>b</sup> | 0.13 <sup>c</sup> | 0.01 | 0.01 | 0.346   | < 0.001 | 0.782  |
| Vh/Cd  | 9.80                  | 11.06 | 10.67 | 10.83 | 12.62 <sup>a</sup>                   | 9.67 <sup>b</sup> | 9.48 <sup>b</sup> | 0.60 | 0.45 | 0.469   | < 0.001 | 0.966  |

Vh villus height, Cd crypt depth, Vh/Cd villus height-to-crypt depth ratio

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

<sup>d</sup>Four dietary treatments: HI0 = control; HI3 = 3% inclusion level of *Hermetia illucens*; HI6 = 6% inclusion level of *Hermetia illucens*; HI9 = 9% inclusion level of *Hermetia illucens*

<sup>e</sup>Three intestinal segments: DU duodenum, JE jejunum, IL ileum

(7.8%, 8.0% and 8.1% of inclusion, respectively), pointed out a higher LW in insect-fed chicks than in the control. In this trial, the FCR was not affected by the dietary treatments, according to the results of Elwert et al. [47] and Cullere et al. [10] pertaining to broiler chickens and broiler quails, respectively, fed with increasing levels of defatted HI meal. On the contrary, an improved FCR was observed in Barbary partridge fed diets with 25% and 50% of substitution of SBM with HI and TM meal [45]. An improved FCR was also reported by Khan et al. [46] (silkworm, housefly maggot, TM) and Bovera et al. [44] (TM), who found that the FCR of broiler chickens was lower in chicks fed insect meal than the control diet with SBM.

The use of HI larva meal as a substitute to corn gluten meal results to be suitable in Muscovy ducks feeding. During the whole experimental period, the growth performances observed in the present study were not influenced by dietary inclusion levels of HI meal, as already observed by other researches in quails and laying hens [10, 24]. Furthermore, the absence of gut histomorphological alterations can also explain the growth performance results, as well as the slightly affected nutrient digestibility.

#### Digestibility trial

The herein obtained results show that the inclusion of HI larva meal in Muscovy duck diets partially affected the apparent digestibility coefficients of the nutrients. Consistently with our results, Cullere et al. [10] did not report any differences in DM and OM total tract apparent digestibility in broiler quails as a result of the HI inclusion level in the diet. On the contrary, in laying hens fed 17% HI meal in the diet, the apparent ileal digestibility of DM was lower in the group fed HI meal than in the control [22]. Results reported by Bovera et al. [24] showed that the inclusion of HI meal by up to 7.3% in laying hen diets did not affect the apparent DM ileal digestibility, compared to the control with vegetable protein meal, whereas the inclusion of 14.6% reduced the apparent DM ileal digestibility. In another study, the use of TM in broiler chicken diets worsened the apparent ileal DM digestibility compared to the control with SBM [44].

In our trial, the CP and EE apparent digestibility in the starter period (3–17 days of age) showed an opposite linear trend, with a reduction in CP digestibility (up to –3.5% in HI9, compared to the control) and an improvement in EE digestibility (+2.0 in HI9, compared to the control) following the increasing inclusion levels of HI meal in the diet. The lower CP digestibility for the HI6 and HI9 groups in the starter period could be related to the higher chitin content of the diet, because of the higher inclusion level of HI meal [48]. In fact, Cutrignelli et al. [22] and Bovera et al. [24] also observed a reduction in the apparent ileal CP digestibility of laying hens compared to the control diet with SBM, and explained this result as a consequence of the presence of chitin in the diet. Indeed, De Marco et al. [9] assumed that the chitin, the structural component of the exoskeleton of insects, can negatively affect the nutrient digestibility, resulting as an indigestible fiber for domestic poultry. However, no differences in CP total tract apparent digestibility were reported by Cullere et al. [10] for broiler quails, after a substitution of protein/fat sources with HI larva meal of up to 15% of inclusion. However, in our trial, the apparent CP digestibility was similar during the grower (18–38 days of age) and finisher (39–50 days of age) periods, thus suggesting an adaptation to the chitin levels in the diet. The studies conducted by Tabata et al. [49, 50] showed that the birds have acid chitinase genes in their genome. In particular, in poultry (such as ducks), the acid chitinase is expressed mainly at the level of the glandular stomach. The level of acid chitinase mRNA in stomach tissue is regulated by feeding behaviour, which was higher in omnivorous species than in herbivorous and carnivorous species [50]. Moreover, it could be speculated that the chitin level in the diet could influence the acid chitinase expression with an overall improvement in feed digestibility.

In our trial, the EE apparent digestibility was higher in HI groups than the control group. However, despite this positive result, the EE apparent digestibility was only 2.2% and 1.9% higher than the control in the starter and grower periods respectively (3–17 and 18–38 days of age) and 3.0% higher than the control in the finisher period (39–50 days of age). This result partially agrees with the results of Cullere et al. [10], who found that the EE total tract

apparent digestibility in broiler quails fed with a 15% inclusion level of HI was higher than a 10% group, but similar to the control diet. On the other hand, Cutrignelli et al. [22] and Bovera et al. [24] found that the apparent ileal EE digestibility in laying hens was similar for HI meal- and SBM-fed birds. The linear increase in EE digestibility is not supported by the fatty acids profile, in particular by the polyunsaturated fatty acids (PUFA) content, that is usually related to an EE digestibility improvement [51]. Indeed, in the present study the dietary PUFA content, as well as the ratio polyunsaturated fatty acids/saturated fatty acids (PUFA/SFA ratio), decreased following the HI larva meal inclusion (Table 1). The highest EE digestibility at the maximum inclusion level (HI9 group) could be related to the overall amount of EE in the diets (+7.84%, +6.06% and +5.19% higher in HI9 group than HI0 group in starter, grower and finisher periods, respectively) [52].

As a whole, the absent or moderate effects on nutrient ATTDC had no impact on the ducks' growth performances, without affecting final LW, ADG, DFI and FCR.

### Histomorphological investigations

Dietary HI meal inclusion did not affect the gut morphology of the ducks of our study. Since the rapid growth of chickens has been reported to strictly depend on the morphological and functional integrity of the digestive tract [25], it is reasonable to hypothesize that insect meal utilization does not negatively influence gut development and, as a consequence, animal performance. The greater mucosal development observed in the duodenum than in the other gut segments is also in agreement with the previous studies available on broilers [11, 14–17, 53, 54], thus suggesting that insect meal utilization leads to the preservation of the physiological intestinal morphology. Indeed, the duodenum is the intestinal tract that undergoes the fastest cell renewal, and is also the first gut segment to receive the physical, chemical and hormonal stimuli caused by the presence of the diet in the lumen [54]. The obtained results about the preservation of gut histomorphology in all dietary groups contributes to validate what has been previously discussed in terms of nutrient digestibility and growth performances.

### Conclusions

To the best of the authors' knowledge, this is the first study that has evaluated the possibility of including insect meal in duck nutrition, and to have demonstrated how HI larva meal can be a valuable protein sources for ducks. Increasing inclusion levels of a partially defatted HI meal in Muscovy duck diets did not affect the growth performances of the birds, which showed similar LW, ADG, DFI and FCR to the control group fed with corn gluten meal, and only had weak effects on the apparent total tract digestibility during the first stages of growth.

Moreover, these increasing levels did not affect the intestinal morphology or cause histopathological alterations. From this preliminary investigation, it appears that HI larva meal can be included in duck feeding at levels of up to 9% of the diet, with no negative effects on growth, digestibility or animal health. Furthermore, the obtained results help to expand the information available about the use of insects in poultry nutrition.

### Abbreviations

ADF: Acid detergent fibre; ADG: Average daily gain; AMEn: Apparent metabolisable energy; ATTDC: Apparent total tract digestibility coefficients; Cd: Crypt depth; CP: Crude protein; DFI: Average daily feed intake; DM: Dry matter; DU: Duodenum; EE: Ether extract; FAME: Fatty acid methyl esters; FCR: Feed conversion ratio; GLMM: General linear mixed model; HE: Haematoxylin & Eosin; HI: *Hermetia illucens*; IL: Ileum; JE: Jejunum; LW: Live weight; MUFA: Monounsaturated fatty acids; NDF: Neutral detergent fiber; OM: Organic matter; PAS: Periodic acid-Schiff; PUFA: Polyunsaturated fatty acids; SB: Sudan Black; SBM: Soybean meal; SEM: Standard error of the mean; SFA: Saturated fatty acids; TM: *Tenebrio molitor*; Vh: Villus height; Vh/Cd: Villus height-to-crypt depth ratio

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### Availability of data and materials

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

### Authors' contributions

MG, SD, FG, LG and AS conceived and designed the experiment. MG, SD, LG, FG, CC, SBO, VR and AS prepared the diets, performed the trial and collected the experimental data. MG, SD, FH, JM and SM performed the digestibility determinations. IB, MTC and EC performed the morphometric investigations. AT established the fatty acids profile. MG, SD, IB and MM performed the statistical analysis. LG, FG, AS, MG, SD, AT analyzed and interpreted the data. LG, MG, SD, IB, AS, FG and AT wrote the first draft of the manuscript. All the authors critically reviewed the intellectual content of the manuscript and gave their approval for the final version to be published.

### Ethics approval and consent to participate

The experimental protocol was approved by the Bioethical Committee of the University of Turin (Italy) (protocol number: 380576, 04/12/2017).

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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Article

# Effects of the Dietary Inclusion of Partially Defatted Black Soldier Fly (*Hermetia illucens*) Meal on the Blood Chemistry and Tissue (Spleen, Liver, Thymus, and Bursa of Fabricius) Histology of Muscovy Ducks (*Cairina moschata domestica*)

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**Simple Summary:** Insects represent a promising feed ingredient for poultry diets, as an alternative to conventional feedstuffs. Black soldier fly (BSF; *Hermetia illucens*) larvae are processed to obtain two main products: the protein and fat fractions. The possible utilization of BSF defatted meal in Muscovy duck (*Cairina moschata domestica*) diets has been poorly investigated. However, its effect on *in vivo* and *post-mortem* traits, which are extremely important for animal welfare, has not yet been investigated. Therefore, the present study has evaluated the effect of 0%, 3%, 6%, and 9% dietary BSF meal replacement on the *in vivo* haematological parameters and on the *post-mortem* organ traits. Overall, the obtained results are encouraging as increasing dietary BSF meal did not impair the growth performance or the haematological traits. Furthermore, both the liver and renal function were unaffected or even improved. The antioxidant picture appeared improved and the histological traits were not influenced by the dietary inclusion of BSF meal. From a productive and biological point of view, the dietary replacement up to 9% of BSF meal in Muscovy duck diet is feasible and BSF meal could represent a promising feed ingredient.

**Abstract:** The present study has evaluated the effects of dietary partially-defatted black soldier fly (BSF; *Hermetia illucens* L.) larva meal on the blood parameters, antioxidant status, and histological features of the organs of broiler ducks. A total of 192 female 3-days of age Muscovy ducklings (*Cairina moschata domestica*) were divided into four dietary treatments (0%, 3%, 6%, and 9% BSF meal inclusion; 6 pens/treatment, 8 birds/pen). A total of 12 ducks/treatment (2 birds/pen) were slaughtered at 51 days of age and blood samples were collected to evaluate the haematological

traits, serum protein, lipid and minerals, liver and renal function serum enzymes, plasma oxidative enzymes, and metabolites. Liver, spleen, thymus, and bursa of Fabricius samples were submitted to histopathological investigations. Between the serum and plasma traits, triglycerides, cholesterol, creatinine, alkaline phosphatase, magnesium, malondialdehyde, and nitrotyrosine showed a linear decrease for increasing amounts of dietary BSF meal ( $p < 0.01$ ); in contrast, the serum iron concentration showed a linear increase ( $p < 0.01$ ). Moreover, the histopathological findings were not significantly affected by the dietary BSF larva meal inclusion. The results showed that the inclusion of up to 9% BSF larva meal represents a promising feed ingredient for Muscovy duck nutrition, and improved blood traits were observed.

**Keywords:** poultry; *Hermetia illucens*; insect meal; blood traits; birds; antioxidant; histology features

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## 1. Introduction

In recent years, researchers have paid a great deal of attention to the use of insect-derived products in poultry [1] and in the nutrition of other monogastric animals, as recently shown in reviews by Sogari et al. [2] and Gasco et al. [3]. The main advantage is the low environmental impact of insects, compared to conventional vegetable protein sources, as they require less soil and water, and lead to lower greenhouse gas and ammonia emissions. Indeed, it has been estimated that 1 ha of land can produce less than 1 ton of soybean protein per year compared to 150 tons of insect protein on the same surface [4]. Similarly, a notable contribution is also given by the limited space requirements of insects, together with their ability to grow on organic wastes, their efficient feed conversion ratio, and their high fecundity [5]. Some researchers have reported that the nutritional value of insects is adequate to support poultry growth, nutrient digestibility, and health [6–9]. However, in other cases, conflicting results have been obtained [10], thus making a thorough study of adequate dietary inclusion levels necessary.

Among the various insect species, black soldier fly (BSF, *Hermetia illucens*) larvae have shown a nutritional composition that is suitable for poultry diets, as it has a high crude protein (CP) content (ranging between 35–57% on a dry matter basis, DM) with a high biological value and an extremely variable ether extract (EE) content (15–49% DM) [11]. The fatty acid composition of BSF larvae depends on the fatty acid composition of the rearing substrate but, generally, the larvae appear rich in lauric acid (20–40% of total lipids), palmitic acid (11–16% of total lipids), and oleic acid (12–32% of total lipids). BSF larvae are also rich in minerals, particularly Ca (5–8% DM) and P (0.6–1.5% DM) [11]. BSF larvae also contain chitin, the main constituent of the exoskeleton of arthropods. The chitin content fluctuates during the life cycle of insects, according to their life stage, but the method used to assess it can lead to dramatic differences in the measurements [12]. The chitin content in BSF larvae ranges from 8.7% [13] to 5.9% [14]. It has been reported that chitin has antioxidant and hypocholesterolemic properties for both humans and animals and appears to have a positive effect on the immune system of poultry, as it exhibits prebiotic properties in the large intestine and appears to exhibit a bacteriostatic effect on Gram-negative bacteria [15–18].

Although the *in vivo* antioxidant effect of chitin has not been investigated in great depth in poultry, it could provide important information about animal welfare. Indeed, the presence of free radicals, especially reactive oxygen species, is associated with several negative biological effects, including deterioration of the DNA, the oxidation of proteins and lipids, and the development of inflammatory disorders [15,19,20]. On the other hand, chitin hypocholesterolemic properties have been mentioned in many studies on broilers and laying hens [16,20,21]. This property could result from the positive charge of this polysaccharide, which binds negatively-charged bile acids and free fatty acids [22]. Finally, the proper functioning and health of the gastrointestinal tract are crucial for ensuring an adequate growth performance in farm animals [23,24]. These aspects are particularly relevant in the poultry industry, where selected birds display an elevated growth potential.

In spite of these interesting biological effects, the use of BSF defatted meal in Muscovy duck (*Cairina moschata domestica*) diets has been poorly investigated so far and, to the best of the authors' knowledge, there is only one paper that shows encouraging results, in terms of growth performance, diet digestibility, and intestinal morphology in ducks [9], while another paper has assessed the *in vitro* digestibility of different insect meals [25].

Considering this background, the present study has been aimed at investigating the effects of dietary BSF larva meal inclusion on the blood parameters and histological traits of female Muscovy ducks, in order to provide a picture of the animal welfare of Muscovy ducks based on a multidisciplinary approach involving both *in vivo* and *post-mortem* parameters.

## 2. Materials and Methods

### 2.1. Birds and Experimental Design

The present trial was performed at the poultry facility of the University of Turin (Italy). The experimental protocol (prot. no. 380576, 4th December 2017) was approved by the Bioethical Committee of the University of Turin (Italy).

The experimental design of the present study is reported in Gariglio et al. [9]. Briefly, a total of 192 female 3-days of age Muscovy ducklings (Canedins R71 L White, Grimaud Freres Selection, France) were divided into four groups, assigned to four different dietary treatments (6 replicates/treatment and 8 birds/replicate) and raised from 3 to 50 days of age. BSF larva meal was included as a substitute for corn gluten meal (substitution 1:1) at increasing levels (0%, 3%, 6%, and 9%; BSF-0, BSF-3, BSF-6, and BSF-9, respectively) in isonitrogenous and isoenergetic diets formulated for three feeding phases: starter (3–17 d), grower (18–38 d), and finisher (39–50 d). In order to evaluate the effects of dietary BSF larva meal inclusion, all the other ingredients were kept constant, with the exception of the synthetic essential amino acids (DL-methionine and L-lysine), as reported in Table 1. The apparent metabolizable energy (AMEn) of the BSF larva meal has previously been assessed for broiler chickens [26] and was used to formulate the diets of this experiment. The diets were formulated to meet or exceed the nutritional requirements of female Muscovy ducks, as reported by Pingel et al. [27].

### 2.2. Chemical Analysis of the BSF Meal and Experimental Diets

Samples of the experimental diets and BSF larva meal were analyzed for DM (AOAC, #934.01), ash (AOAC, #942.05), CP (AOAC, #984.13), EE (AOAC, #2003.05), neutral detergent fiber (NDF) (AOAC, #2002.04), and acid detergent fiber (ADF) (AOAC, #973.18) [28,29]. The method of Finke et al. [30] was used for the determination of the chitin content of the BSF larva meal using ADF adjusted for its nitrogen content. The chemical composition of the experimental diets is reported in Table 1. Moreover, the chemical composition of the BSF larva meal (on a DM basis) was as follows: 924.1 g/kg DM, 567.1 g/kg CP, 107.0 g/kg EE, 163.8 g/kg ash, and 64.3 g/kg chitin.

**Table 1.** Ingredients (g/kg as fed) and nutrient composition (%) of the experimental diets.

| Ingredients                  | Starter period (3 to 17 d) <sup>1</sup> |       |       |       | Grower period (18 to 38 d) <sup>1</sup> |       |       |       | Finisher period (39 to 50 d) <sup>1</sup> |       |       |       |
|------------------------------|-----------------------------------------|-------|-------|-------|-----------------------------------------|-------|-------|-------|-------------------------------------------|-------|-------|-------|
|                              | BSF-0                                   | BSF-3 | BSF-6 | BSF-9 | BSF-0                                   | BSF-3 | BSF-6 | BSF-9 | BSF-0                                     | BSF-3 | BSF-6 | BSF-9 |
| Corn meal                    | 600.0                                   | 600.0 | 600.0 | 600.0 | 638.0                                   | 638.0 | 638.0 | 638.0 | 670.0                                     | 670.0 | 670.0 | 670.0 |
| Soybean meal                 | 212.0                                   | 212.0 | 212.0 | 212.0 | 160.0                                   | 160.0 | 160.0 | 160.0 | 100.0                                     | 100.0 | 100.0 | 100.0 |
| BSF larva meal               | 0.0                                     | 30.0  | 60.0  | 90.0  | 0.0                                     | 30.0  | 60.0  | 90.0  | 0.0                                       | 30.0  | 60.0  | 90.0  |
| Bran                         | 42.5                                    | 42.5  | 42.5  | 42.5  | 36.3                                    | 36.3  | 36.3  | 36.3  | 66.2                                      | 66.2  | 66.2  | 66.2  |
| Corn gluten meal             | 90.0                                    | 60.0  | 30.0  | 0.0   | 90.0                                    | 60.0  | 30.0  | 0.0   | 90.0                                      | 60.0  | 30.0  | 0.0   |
| Soybean oil                  | 16.5                                    | 16.5  | 16.5  | 16.5  | 28.5                                    | 28.5  | 28.5  | 28.5  | 34.5                                      | 34.5  | 34.5  | 34.5  |
| Dicalcium phosphate          | 10.0                                    | 10.0  | 10.0  | 10.0  | 13.0                                    | 13.0  | 13.0  | 13.0  | 4.0                                       | 4.0   | 4.0   | 4.0   |
| Calcium carbonate            | 8.0                                     | 8.0   | 8.0   | 8.0   | 14.0                                    | 14.0  | 14.0  | 14.0  | 17.4                                      | 17.4  | 17.4  | 17.4  |
| Sodium chloride              | 2.5                                     | 2.5   | 2.5   | 2.5   | 2.5                                     | 2.5   | 2.5   | 2.5   | 2.5                                       | 2.5   | 2.5   | 2.5   |
| Sodium bicarbonate           | 2.0                                     | 2.0   | 2.0   | 2.0   | 2.0                                     | 2.0   | 2.0   | 2.0   | 2.0                                       | 2.0   | 2.0   | 2.0   |
| DL-methionine                | 2.5                                     | 2.5   | 2.6   | 2.8   | 1.7                                     | 1.8   | 1.9   | 2.2   | 0.3                                       | 0.4   | 0.5   | 0.8   |
| L-lysine                     | 3.9                                     | 3.9   | 3.8   | 3.6   | 3.9                                     | 3.8   | 3.7   | 3.4   | 3.0                                       | 2.9   | 2.8   | 2.5   |
| MinVit premix <sup>2</sup>   | 5.0                                     | 5.0   | 5.0   | 5.0   | 5.0                                     | 5.0   | 5.0   | 5.0   | 5.0                                       | 5.0   | 5.0   | 5.0   |
| Choline chloride             | 0.1                                     | 0.1   | 0.1   | 0.1   | 0.1                                     | 0.1   | 0.1   | 0.1   | 0.1                                       | 0.1   | 0.1   | 0.1   |
| Optifos 250 bro <sup>3</sup> | 1.0                                     | 1.0   | 1.0   | 1.0   | 1.0                                     | 1.0   | 1.0   | 1.0   | 1.0                                       | 1.0   | 1.0   | 1.0   |
| Avizyme 1500 x <sup>4</sup>  | 1.0                                     | 1.0   | 1.0   | 1.0   | 1.0                                     | 1.0   | 1.0   | 1.0   | 1.0                                       | 1.0   | 1.0   | 1.0   |
| Titanium dioxide             | 3.0                                     | 3.0   | 3.0   | 3.0   | 3.0                                     | 3.0   | 3.0   | 3.0   | 3.0                                       | 3.0   | 3.0   | 3.0   |
| Total                        | 1000                                    | 1000  | 1000  | 1000  | 1000                                    | 1000  | 1000  | 1000  | 1000                                      | 1000  | 1000  | 1000  |
| AMEn, MJ/kg                  | 12.12                                   | 12.10 | 12.08 | 12.07 | 12.53                                   | 12.51 | 12.49 | 12.47 | 12.77                                     | 12.75 | 12.74 | 12.72 |
| Nutrient composition         |                                         |       |       |       |                                         |       |       |       |                                           |       |       |       |
| DM                           | 89.3                                    | 89.8  | 90.1  | 89.7  | 88.8                                    | 89.2  | 89.1  | 89.1  | 88.7                                      | 89.0  | 89.3  | 88.9  |
| CP, % DM                     | 25.1                                    | 24.7  | 25.2  | 24.8  | 23.0                                    | 22.5  | 22.5  | 22.4  | 20.2                                      | 20.2  | 20.1  | 20.1  |
| EE, % DM                     | 4.7                                     | 4.8   | 4.9   | 5.1   | 6.2                                     | 6.2   | 6.4   | 6.6   | 7.3                                       | 7.4   | 7.6   | 7.7   |
| NDF, % DM                    | 12.7                                    | 12.3  | 12.7  | 12.4  | 12.9                                    | 13.1  | 12.6  | 12.7  | 12.7                                      | 13.1  | 12.7  | 12.9  |
| ADF, % DM                    | 3.4                                     | 3.5   | 3.7   | 3.3   | 3.5                                     | 3.5   | 3.7   | 3.5   | 3.4                                       | 3.5   | 3.5   | 3.4   |
| Ash, % DM                    | 5.6                                     | 6.0   | 5.6   | 5.8   | 7.8                                     | 7.5   | 7.5   | 8.0   | 6.5                                       | 6.4   | 6.9   | 6.7   |

Note: BSF: black soldier fly; AMEn: apparent metabolisable energy; DM: dry matter; CP: crude protein; EE: ether extract; NDF: neutral detergent fiber; ADF: apparent digestible fiber. <sup>1</sup>BSF-0, BSF-3, BSF-6, and BSF-9 = dietary inclusion of BSF larva meal at 0%, 3%, 6%, and 9%, respectively. <sup>2</sup>Mineral-vitamin premix. Composition per kg of diet: vitamin A (retinyl acetate), 62.5 IU; vitamin D3 (cholecalciferol), 17.5 IU; vitamin E (DL- $\alpha$ -tocopheryl acetate), 200  $\mu$ g; vitamin K (menadione sodium bisulfite), 10  $\mu$ g; biotin, 1  $\mu$ g; thiamine, 10  $\mu$ g; riboflavin, 30  $\mu$ g; pantothenate, 76.05  $\mu$ g; niacin, 200  $\mu$ g; choline, 3750  $\mu$ g; pyridoxine, 20  $\mu$ g; folic acid, 3.75  $\mu$ g; vitamin B12, 0.15  $\mu$ g; Mn, 350  $\mu$ g; Zn, 310.75  $\mu$ g; Fe, 250  $\mu$ g; Cu, 350  $\mu$ g; I, 1.25  $\mu$ g; Se, 1.25  $\mu$ g. <sup>3</sup>Optifos 250 bro: Phytase (EC 3.1.3.26) (250 OTU/kg diet), Huvepharma, Sofia, Bulgaria. <sup>4</sup>Avizyme 1500 x: Complex of Endo 1-4-Beta- Xylanase (EC 3.2.1.8) (256 U/kg), subtilisine (Ec 3.4.21.62) (2560 U/kg diet) and alpha-amylase (EC3.2.1.1) (1472 U/kg diet), Danisco Animal Nutrition, Marlborough, Wiltshire, UK.

### 2.3. Growth Performance

The growth performance parameters were evaluated, as previously reported by Gariglio et al. [9]. The live weight (LW) of the birds was assessed at the beginning and at the end of the trial (at 3 and 50 days of age, respectively), and the average daily gain (ADG), the daily feed intake (DFI), and the feed conversion ratio (FCR) were calculated for the whole experimental period (3–50 days of age). The mortality and health status of birds were monitored on a daily basis.

### 2.4. Slaughtering Procedures and Sampling

At 50 days of age, 12 ducks per diet (two birds per pen) were selected on the basis of the average LW and identified through a shank ring. Subsequently, after a feed withdrawal period of 12 hours (at 51 days of age), the selected ducks were transferred to a commercial processing plant and slaughtered according to the standard EU regulations.

At slaughtering, blood samples were collected in EDTA tubes and serum-separating tubes, further details of which are provided in Section 2.5.

Immediately after the completion of the slaughtering phase, spleen, liver, thymus, and bursa of Fabricius samples were collected and fixed in a 10% buffered formalin solution for histochemical staining, further details of which are provided in Section 2.6.

### 2.5. Blood Analysis

Blood samples were collected, at slaughtering, from the jugular vein of twelve birds (two animals per pen) per feeding group. An aliquot of 2.5 mL was placed in an EDTA tube and 2.5 mL in a serum-separating tube. A blood smear was prepared from a droplet without any anticoagulant. The total red (erythrocytes) and white (leukocytes) cell counts were determined in an improved Neubauer haemocytometer after mixing with a Natt-Herrick solution in a 1 to 200 ratio, as reported by Natt and Herrick [31]. The blood smears were stained with May-Grünwald and Giemsa–Romanowski stains. One hundred white blood cells were evaluated per smear to determine the heterophils to lymphocytes (H/L) ratio, while the number of blood cell types was determined according to Campbell [32].

The serum-separating tubes were left in a standing position, at room temperature, for approximately two hours, until the formation of a blood clot. Subsequently, the tubes were centrifugated at  $700\times g$  for 15 minutes and the obtained serum was immediately frozen at  $-80\text{ }^{\circ}\text{C}$ . The total protein was quantified using the “biuret method” (Bio Group Medical System kit; Bio Group Medical System, Talamello (RN), Italy); the electrophoretic pattern of the serum was assessed using a semi-automated agarose gel electrophoresis system (Sebia Hydrasys®, Norcross, GA, USA). The alanino-aminotransferase (ALT), aspartate-aminotransferase (AST), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), triglycerides, cholesterol, Ca, P, Mg, Fe, uric acid, and creatinine serum concentrations were measured using enzymatic methods in a clinical chemistry analyzer (Screen Master Touch, Hospitex diagnostics Srl., Firenze, Italy), as described by Salamano et al. [33].

In order to obtain plasma, the EDTA tubes were centrifugated at  $2000\times g$  for 10 minutes to separate the cell fractions, and the supernatants were immediately frozen at  $-80\text{ }^{\circ}\text{C}$  and then used to determine the antioxidant status and oxidative metabolites. The blood glutathione peroxidase (GPx, EC 1.11.1.9) and total antioxidant status (TAS) activities of the plasma were determined using a Ransel Enzymatic Kit (RS504, Randox Laboratories, Crumlin, UK) and a TAS Colorimetric Kit (NX2332, Randox Laboratories, Crumlin, UK), respectively, according to the manufacturer’s recommendations.

The enzyme immunoassay for the detection and quantification of methylglyoxal (MG) was performed using an OxiSelect™ Methylglyoxal ELISA Kit (STA-811, Cell Biolabs, San Diego, CA, USA), while an OxiSelect™ MDA Adduct Competitive ELISA Kit (STA-832, Cell Biolabs, San Diego, CA, USA) was used for the malondialdehyde (MDA) quantification. Both tests were performed on plasma samples. The 3-nitrotyrosine plasma concentration was measured by an enzyme-linked

immunosorbent assay (ELISA) using an OxiSelect™ Nitrotyrosine ELISA Kit (STA-305, Cell Biolabs, San Diego, CA, USA). All the tests were performed according to the manufacturer's instructions.

All the analyses were performed in duplicate.

## 2.6. Histological Investigations

The slaughtered birds (n = 12 per experimental diet, two birds per pen) were submitted to an anatomopathological examination. Spleen (entire organ), liver (left lobe), thymus (left side lobes), and bursa of Fabricius (entire organ) samples were collected (0.5–1.5 g/organ) and fixed in a 10% buffered formalin solution, embedded in paraffin wax blocks, sectioned at a thickness of 5 µm, mounted onto glass slides, and stained with Haematoxylin & Eosin for the histopathological examination [34]. The following histopathological alterations were evaluated: white pulp hyperplasia and depletion in the spleen, cortical depletion in the thymus, follicular depletion and intrafollicular cysts in the bursa of Fabricius, and hepatocyte degeneration and lymphoid tissue activation in the liver [6]. The observed histopathological alterations were evaluated using a semiquantitative scoring system as follows: absent (score = 0), mild (score = 1), moderate (score = 2), and severe (score = 3). In order to investigate the accumulation of lipids and polysaccharides in the liver, tissue samples of these organs were also stained with Sudan Black and Periodic acid-Schiff (PAS), respectively. The lipid and polysaccharide staining intensity was scored semi-quantitatively as follows: grade 0 for an absence of staining, grade 1 for mild staining, grade 2 for moderate staining, and grade 3 for marked staining. All the slides were blindly evaluated by three different observers and the discordant cases were reviewed using a multi-head microscope until a unanimous consensus had been reached.

## 2.7. Statistical Analysis

The statistical analysis was performed using the SPSS software package (version 21 for Windows, SPSS Inc., Chicago, IL, USA). The mortality rate was analyzed by means of a Chi-square test, using the BSF0 group as reference. Shapiro-Wilk's test was used to establish the normality or non-normality of the distributions. The assumption of equal variances was assessed by means of Levene's homogeneity of variance test. The birds' pen was identified as the experimental unit to evaluate the growth performance, while the blood traits and histological features were evaluated individually for each duck. The collected data were tested using one-way ANOVA. Polynomial contrasts were used to test the linear and quadratic responses to increased levels of BSF inclusion in the diet. Histopathological scores were analyzed by means of the Kruskal-Wallis test (post-hoc test: Dunn's Multiple Comparison test). Differences between treatments were considered statistically significant when the  $p$  values  $\leq 0.05$ .

## 3. Results

### 3.1. Growth Performance

The growth performance of the broiler ducks, as reported in detail by Gariglio et al. [9], is summarized in Table 2. Overall, LW, ADG, DFI, and FCR were not influenced by the dietary treatments ( $p > 0.05$ ). The cumulative mortality rates were 4.16 % (for BSF-0) and 2.08% (for BSF-3, BSF-6, and BSF-9) and no statistical effect of dietary treatment was found for this parameter ( $p > 0.05$ ).

**Table 2.** Growth performance of Muscovy ducks fed increasing BSF meal from 3 to 50 d of age (n = 6 pen; 8 birds/pen).

| Items    | Age    | Dietary Treatments <sup>1</sup> |        |        |        | SEM   | <i>p</i> -value |           |
|----------|--------|---------------------------------|--------|--------|--------|-------|-----------------|-----------|
|          |        | BSF-0                           | BSF-3  | BSF-6  | BSF-9  |       | Linear          | Quadratic |
| LW, g    | 3 d    | 70.7                            | 70.4   | 72.6   | 71.5   | 0.60  | 0.405           | 0.733     |
| LW, g    | 50 d   | 2540.6                          | 2511.1 | 2456.1 | 2554.8 | 20.13 | 0.946           | 0.123     |
| ADG, g/d | 3–50 d | 52.5                            | 51.9   | 50.7   | 52.8   | 0.43  | 0.926           | 0.125     |
| DFI, g/d | 3–50 d | 120.6                           | 121.3  | 117.6  | 121.5  | 1.20  | 0.927           | 0.530     |
| FCR, g/g | 3–50 d | 2.29                            | 2.34   | 2.32   | 2.30   | 0.019 | 0.925           | 0.406     |

Note: BSF: black soldier fly; SEM: standard error of the mean; LW: live weight; ADG: average daily gain; DFI: daily feed intake; FCR: feed conversion ratio. <sup>1</sup>BSF-0, BSF-3, BSF-6, and BSF-9 = dietary inclusion of BSF larva meal at 0%, 3%, 6%, and 9%, respectively.

### 3.2. Blood Traits

As reported in Table 3, the haematological traits of the Muscovy ducks fed up to 9% dietary BSF larva meal were not influenced by the dietary treatments. Serum protein was similar between groups, while the triglycerides and cholesterol levels displayed a linear decrease ( $p < 0.05$ ), as a result of increasing levels of dietary BSF larva meal (30.23% and 23.86% lower in BSF9 than BSF0, respectively; Table 3).

**Table 3.** Haematological traits and the serum protein and lipids of Muscovy ducks fed increasing BSF larva meal from 3 to 50 d of age (n = 12 birds/dietary treatment).

| Items                                         | Dietary Treatments <sup>1</sup> |       |       |       | SEM  | <i>p</i> -value |           |
|-----------------------------------------------|---------------------------------|-------|-------|-------|------|-----------------|-----------|
|                                               | BSF-0                           | BSF-3 | BSF-6 | BSF-9 |      | Linear          | Quadratic |
| Haematological Traits                         |                                 |       |       |       |      |                 |           |
| Erythrocytes, 10 <sup>6</sup> , cell/ $\mu$ L | 5.39                            | 4.98  | 4.98  | 4.92  | 0.11 | 0.160           | 0.429     |
| Leukocytes, 10 <sup>3</sup> , cell/ $\mu$ L   | 17.58                           | 17.52 | 18.08 | 18.03 | 0.34 | 0.547           | 0.991     |
| Heterophils, %                                | 50.83                           | 51.75 | 46.18 | 51.16 | 1.31 | 0.697           | 0.443     |
| Lymphocytes, %                                | 47.75                           | 46.17 | 50.91 | 46.58 | 1.36 | 0.920           | 0.623     |
| Monocytes, %                                  | 0.58                            | 1.00  | 1.09  | 0.83  | 0.14 | 0.501           | 0.235     |
| Eosinophils, %                                | 0.33                            | 0.75  | 1.18  | 1.17  | 0.19 | 0.092           | 0.576     |
| Basophils, %                                  | 0.50                            | 0.33  | 0.64  | 0.25  | 0.12 | 0.673           | 0.646     |
| H/L                                           | 0.98                            | 0.96  | 0.96  | 0.97  | 0.03 | 0.997           | 0.876     |
| Serum proteins and lipids                     |                                 |       |       |       |      |                 |           |
| Total Protein, g/dl                           | 4.26                            | 4.84  | 4.99  | 4.79  | 0.11 | 0.086           | 0.082     |
| Triglycerides, mg/dl                          | 73.27                           | 58.38 | 55.93 | 51.12 | 3.08 | 0.012           | 0.395     |
| Cholesterol, mg/dl                            | 90.79                           | 85.25 | 82.71 | 69.13 | 3.13 | 0.016           | 0.507     |

Note: BSF: black soldier fly; SEM: standard error of the mean; H/L: Heterophils to lymphocytes ratio. <sup>1</sup>BSF-0, BSF-3, BSF-6, and BSF-9 = dietary inclusion of BSF larva meal at 0%, 3%, 6%, and 9%, respectively.

Table 4 reports the results for the serum minerals and for the liver and renal function parameters. Ca and P were unaffected by the dietary treatments, while a linear response was observed for the Mg and Fe concentrations. In particular, Mg showed a linear decrease as a result of the increasing dietary inclusion levels of BSF larva meal, while Fe showed a linear increase, with a maximum for the BSF-9 group ( $p < 0.01$ ).

The parameters associated with the liver functions, including AST, ALT, and GGT, were unaffected for all the groups, while ALP showed a linear decrease ( $p < 0.01$ ).

The renal function was partially influenced by the dietary inclusion of BSF larva meal. No changes in the levels of uric acid were observed. However, a linear decrease in the creatinine values ( $p < 0.05$ ) was identified.

As reported in Table 5, the antioxidant enzymes and oxidative metabolites picture was partially influenced by the dietary treatments. GPx, TAS, and MG remained constant between groups, while MDA and nitrotyrosine showed a linear decrease with increasing dietary levels of BSF ( $p < 0.001$ ).

**Table 4.** Serum minerals and the liver and renal functions of Muscovy ducks fed increasing levels of BSF larva meal from 3 to 50 d of age (n = 12 birds/dietary treatment).

| Items             | Dietary Treatments <sup>1</sup> |        |        |        | SEM   | p-value |           |
|-------------------|---------------------------------|--------|--------|--------|-------|---------|-----------|
|                   | BSF-0                           | BSF-3  | BSF-6  | BSF-9  |       | Linear  | Quadratic |
| Minerals          |                                 |        |        |        |       |         |           |
| Ca, mg/dl         | 10.02                           | 10.59  | 10.56  | 9.27   | 0.37  | 0.469   | 0.840     |
| P, mg/dl          | 3.87                            | 4.35   | 3.99   | 3.93   | 0.16  | 0.900   | 0.407     |
| Mg, mg/dl         | 1.48                            | 1.43   | 1.32   | 1.25   | 0.03  | 0.002   | 0.858     |
| Fe, mg/l          | 310.22                          | 327.33 | 371.18 | 406.39 | 13.80 | 0.007   | 0.731     |
| Liver function    |                                 |        |        |        |       |         |           |
| AST, U/l          | 27.14                           | 27.32  | 27.08  | 28.58  | 0.89  | 0.622   | 0.717     |
| ALT, U/l          | 27.54                           | 30.49  | 29.48  | 25.41  | 1.30  | 0.530   | 0.187     |
| GGT, U/l          | 4.83                            | 4.23   | 4.82   | 4.92   | 0.23  | 0.685   | 0.452     |
| ALP, U/l          | 2003.6                          | 1889.8 | 1876.4 | 1831.1 | 22.59 | 0.008   | 0.426     |
| Renal function    |                                 |        |        |        |       |         |           |
| Uric acid, mg/dl  | 3.98                            | 3.88   | 3.69   | 3.31   | 0.17  | 0.152   | 0.679     |
| Creatinine, mg/dl | 0.30                            | 0.29   | 0.29   | 0.25   | 0.01  | 0.022   | 0.526     |

Note: BSF: black soldier fly; SEM: standard error of the mean; Ca: calcium; P: phosphorus; Mg: magnesium; Fe: iron; AST: aspartate-aminotransferase; ALT: alanine-aminotransferase; GGT: gamma-glutamyl transferase; ALP: alkaline phosphatase. <sup>1</sup>BSF-0, BSF-3, BSF-6, and BSF-9 = dietary inclusion of BSF larva meal at 0%, 3%, 6%, and 9%, respectively.

**Table 5.** Antioxidant enzymes and oxidative metabolites of the Muscovy ducks fed increasing levels of BSF larva meal from 3 to 50 d of age (n = 12 birds/dietary treatment).

| Items             | Dietary treatments <sup>1</sup> |        |        |        | SEM   | p-value |           |
|-------------------|---------------------------------|--------|--------|--------|-------|---------|-----------|
|                   | BSF-0                           | BSF-3  | BSF-6  | BSF-9  |       | Linear  | Quadratic |
| GPx, U/g Hb       | 216.54                          | 240.27 | 229.74 | 246.39 | 7.51  | 0.250   | 0.816     |
| TAS, mmol/l       | 1.48                            | 1.47   | 1.46   | 1.43   | 0.03  | 0.618   | 0.870     |
| MG, µg/ml         | 0.49                            | 0.50   | 0.47   | 0.46   | 0.01  | 0.307   | 0.747     |
| MDA, pmol/ml      | 161.99                          | 169.97 | 148.73 | 147.10 | 2.10  | 0.000   | 0.152     |
| Nitrotyrosine, nM | 193.10                          | 133.42 | 122.66 | 78.67  | 11.01 | 0.000   | 0.677     |

Note: BSF: black soldier fly; SEM: standard error of the mean; GPx: glutathione peroxidase; Hb: haemoglobin; TAS: total antioxidant status; MG: methylglyoxal; MDA: malondialdehyde. <sup>1</sup>BSF-0, BSF-3, BSF-6, and BSF-9 = dietary inclusion of BSF larva meal at 0%, 3%, 6%, and 9%, respectively.

### 3.3. Histological Investigations

No macroscopic lesions were detected during the anatomopathological examination in any of the slaughtered birds. Histopathological changes were identified in all the examined organs collected from the birds belonging to all the dietary treatments. Regardless of the dietary treatment, the spleen showed mild, multifocal white pulp hyperplasia (range score: 0.20–0.42), while mild, multifocal cortical depletion was observed in the thymus (range score: 0.00–0.18). Furthermore, mild to moderate, multifocal to diffuse follicular depletion was detected in the bursa of Fabricius (range score: 0.00–0.36), whereas the liver showed mild to severe, multifocal to diffuse steatosis (mild to marked SB staining intensity) or vacuolar degeneration of the hepatocytes (range score: 0.77–1.88). No polysaccharide accumulation was observed in the hepatocytes (absence of PAS positivity). The dietary BSF larva meal inclusion did not affect the severity of the observed histopathological changes ( $p > 0.05$ , Table 6).

**Table 6.** Histopathological scores of the Muscovy ducks fed increasing BSF larva meal from 3 to 50 d of age (n = 12 birds/dietary treatment).

| Items              | Dietary Treatments <sup>1</sup> |       |       |       | SEM  | p-value |
|--------------------|---------------------------------|-------|-------|-------|------|---------|
|                    | BSF-0                           | BSF-3 | BSF-6 | BSF-9 |      |         |
| Spleen             | 0.42                            | 0.29  | 0.27  | 0.20  | 0.07 | 0.740   |
| Liver              | 1.38                            | 1.88  | 0.77  | 1.38  | 0.15 | 0.096   |
| Thymus             | 0.10                            | 0.18  | 0.09  | 0.00  | 0.06 | 0.549   |
| Bursa of Fabricius | 0.33                            | 0.25  | 0.00  | 0.36  | 0.07 | 0.224   |

Note: BSF: black soldier fly; SEM: standard error of the mean. <sup>1</sup>BSF-0, BSF-3, BSF-6, and BSF-9 = dietary inclusion of BSF larva meal at 0%, 3%, 6%, and 9%, respectively.

## 4. Discussion

### 4.1. Growth Performance

The first part of the study evaluated the use of BSF larva meal in Muscovy ducks from a nutritional point of view [9], and this was followed by the investigation of the blood chemistry and tissue histology reported in this paper. The percentages of BSF larva meal included in the feeds used in the study appear compatible with the digestive physiology of the Muscovy duck, since no detrimental effects on growth, digestibility, or intestinal morphology were noted [9]. A low mortality rate and a lack of clinical signs indicates that the inclusion of low levels of dietary BSF larva meal does not have any detrimental effects on the health of the birds. This is further supported by the results of the blood chemistry investigation and tissue histological findings discussed below.

### 4.2. Blood Traits

The blood parameters considered in this study provide an overview of the health status and the welfare of birds. The unaffected haematological picture, as well as the H/L ratio (which is related to the presence of stressful conditions [35]), support the lack of stress resulting from the use of BSF larva meal in Muscovy duck diets. These results are in line with what has been reported for broiler chickens [10], laying hens [16,21], and Barbary partridges [7]. In addition, the plasma protein levels were similar between groups, thus suggesting a similar dietary protein bioavailability. However, the blood triglycerides and cholesterol levels appeared reduced as the dietary inclusion levels of BSF larva meal were increased, as has also been described for laying hens [16,21]. A possible explanation of this effect could be due to the chelating effect of chitin in the BSF larva meal [22,23,36].

In our study, Ca and P were not influenced by the dietary treatments. However, Mg showed a linear decrease and Fe a linear increase. The results obtained in the present study can be contextualized within the wide variability of the findings reported in other studies [7,10,16,21].

The functioning of the organs appeared preserved or improved in all the experimental groups, as shown by the liver and renal serum enzyme levels. Indeed, the liver enzymes, as confirmed by the ALT, AST, and GGT concentrations, remained constant between treatments. In addition, ALP was found to be lower in the insect-fed groups than the BSF-0 (8.61% lower in BSF-9 than BSF-0), thus demonstrating the absence of adverse effects of dietary BSF larva meal inclusion on the liver function of Muscovy ducks, as previously demonstrated for broiler chickens [10]. On the other hand, the unchanged uric acid levels and the linear decrease in the creatinine levels showed a partially improved renal function. This result is in line with what Marono et al. [16] observed for laying hens. Although chitin is not associated with renal functioning, some papers have reported that chitosan, produced by chitin deacetylation, was able to improve the renal function of rats and humans, and that they showed decreased serum creatinine levels [37–39].

In the present paper, the blood and plasma antioxidant enzymes (GPx and TAS) were not affected by the BSF larva meal inclusion levels. On the other hand, the plasma oxidative metabolites were affected by the dietary treatments, and showed decreased values of MDA and nitrotyrosine as a result

of the dietary inclusion levels of BSF larva meal. The MDA is the end product of lipid breakdown, as a result of oxidative stress [40]. Furthermore, the lipid peroxidation process involves the oxidation of tyrosine with the formation of nitrotyrosine, which promotes the oxidative tissue damage [41]. Increased levels of 3-nitrotyrosine have been correlated with high levels of other indices of oxidative stress markers associated with apoptotic cell death [42,43]. Although nitrotyrosine is a commonly evaluated parameter in humans and rats [44,45], only a few studies have conducted an assessment of nitrotyrosine as an oxidative stress marker in poultry [46,47]. Although the positive effects of dietary antioxidants have been investigated extensively in Muscovy ducks [48–50], the antioxidant effect of chitin has not yet been elucidated. The potential free radical scavenging effect of chitin may play an important role in animal health by preserving cellular integrity and the normal biological functions.

#### 4.3. Histological Investigations

The dietary BSF larva meal inclusion did not significantly influence either the development or severity of the histopathological alterations detected in the ducks of the current research. This is in agreement with Biasato et al. [6,51,52] and Dabbou et al. [10], who did not find any significant alterations in broiler chickens fed diets with yellow mealworm (*Tenebrio molitor*, TM) or BSF inclusions, respectively. Regardless of the dietary treatment, the ducks of the four experimental groups showed more severe hepatic lesions than those recorded in broiler chickens fed TM meal [6,51,52] or BSF meal [10]. This difference can be explained by considering the genetic predisposition of ducks to developing hepatic steatosis under intensive farming conditions [53,54]. Furthermore, Chartrin et al. [55] and Hérault et al. [56] reported that Muscovy ducks showed a greater tendency to accumulate lipids in the liver than Pekin ducks. Lipid mobilization from the liver to the peripheral tissues is generally related to the availability of transport lipoproteins [57]. On the basis of these findings, it is possible to state that the BSF larva meal showed no negative effects on animal health.

## 5. Conclusions

Our study provides original and novel information about the use of BSF larva meal in the nutrition of Muscovy ducks. Increasing the dietary levels of BSF larva meal did not impair the growth performance or haematological traits of the birds. Furthermore, both the liver and renal functions were unaffected or even improved. According to the literature, the serum minerals have provided conflicting results, thus underlying the need for additional studies on this topic. The antioxidant picture was improved, and the histological traits were not influenced by the addition of the dietary BSF larva meal. Moreover, some metabolites (MG and nitrotyrosine) were evaluated for the first time in Muscovy ducks. From a productive and biological point of view, the BSF larva meal, at inclusion levels of up to 9%, represents a promising feed ingredient for the nutrition of Muscovy ducks.

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