Effects of different levels of *Hermetia illucens* larvae meal on performance, egg quality, yolk fatty acid composition and oxidative status of laying hens

Xu Liu\(^a\), Xin Liu\(^a\), Yaling Yao\(^b\), Xiangyong Qu\(^a\), Jifa Chen\(^c\), Kailai Xie\(^a\), Xingju Wang\(^a\), Yi Qi\(^a\), Bing Xiao\(^d\) and Changqing He\(^a\)

\(^a\)Hunan Engineering Research Center of Poultry Production Safety, Hunan Co-Innovation Center of Animal Production Safety, College of Animal Science and Technology, Hunan Agricultural University, Changsha, China; \(^b\)Huaihua Animal Husbandry and Fishery Affairs Center, Huaihua, China; \(^c\)College of Life Science and Resources and Environment, Yichun University, Yichun, P. R. China; \(^d\)Hunan Yunfeifeng Agricultural Co. Ltd, Huaihua, China

**ABSTRACT**

To investigate the supplemental effects of *Hermetia illucens* larvae meal (HILM) on the production, egg quality, yolk fatty acid composition, egg amino acid content, oxidative status, and immune indexes of laying hens. A total of 432 Xuefeng black-bone chickens (45-week-old) were randomly assigned to 4 treatments with 6 replicates per treatment and 18 hens in each replicate. The laying hens were fed the basal diet supplemented with 0%, 1%, 3%, and 5% HILM (CON, HILM-1, HILM-3, and HILM-5, respectively) for 56 d. The experimental diets were isonitrogenous and isoenergetic. The highest egg weight and Haugh unit were obtained with 3% HILM supplementation, but the HILM-3 or HILM-5 supplementation have negative effect on the eggshell thickness, yolk index, and albumen height during the early stage of the experiment. The HILM supplementation linearly or quadratically \((p < .05)\) increased the C14:0, C17:0, and C20:2 contents of yolk, the Glu, Val, Met, Phe, and Leu contents of egg, the T-SOD and CAT activities, and the NDV-Ab and AIV-Ab levels of plasma. With increasing supplementation of HILM in the basal diet, FCR, the C16:0 and C16:1 contents of yolk, MDA content and IL-2 level of plasma decreased both linearly or quadratically \((p < .05)\). In conclusion, *Hermetia illucens* larvae meal can be a suitable alternative protein source for Xuefeng black-bone chicken. Dietary supplementation of 3% HILM in basal diets may be a feasible means of effectively increasing the production performance of laying hens, and partially enhanced hens’ antioxidant capability and immune function.

**HIGHLIGHTS**

- Supplementation of 1% HILM had no effect on laying hens, and supplementation of 3% HILM increased the egg weight and Haugh unit.
- Supplementation of 3% HILM or 5% HILM have a negative effect on the eggshell thickness, yolk index, and albumen height during the early stage of the experiment.
- Supplementation of 3% HILM increased the C14:0, C17:0, and C20:2 contents of yolk, the Glu, Val, Met, Phe, and Leu contents of egg.
- The plasma antioxidant status and immune response improved by dietary addition of HILM at 3% levels.

**Introduction**

As a result of the expected increase in the global human population, the worldwide demand for animal protein is expected to be 58% higher in 2050 than its level in 2010 (FAO 2013). At the same time, the phenomenon of competition between human and livestock for food will become increasingly prominent, especially the demand for protein feed (Makkar et al. 2014). Soybean meal and fish meal are the traditional and essential protein feed resources. However, the global land availability for soy cultivation is limited and marine overexploitation has reduced the abundance of small pelagic forage fish from which fish meal and fish oil are derived (Veldkamp and Bosch 2015). Therefore, it is necessary to find a suitable and economical alternative protein source for human and animal nutrition.

Xuefeng black-bone chicken was originated from the Xuefeng mountain area in the southwest of China.
Hunan Province and has been bred naturally for a long time. It is not only China’s meat and egg-type silky chicken breed, but also a valuable rare bird. In January 2010, Xuefeng black-bone chicken was included in the List of National Livestock and Poultry Genetic Resources Protection in China (Liu et al. 2019). Insects, the largest group of organisms on earth, have high protein and lipid contents and can be reared on organic waste substrates with high feed conversion efficiency (Van Huis 2013; Veldkamp and Bosch 2015) and fecundity (Nakagaki and Defoliart 1991), thus enabling a ‘circular bioeconomy’. As one of the most promising novel protein sources in animal feeds, insects have already been used to feed fish (Magalhães et al. 2017; Renna et al. 2017), pigs (Biasato et al. 2019; Yu et al. 2019), and poultry (Cullere et al. 2016; Marono et al. 2017; Józefiak et al. 2018). Compared with other insects, Hermetia illucens larvae meal (HILM) is considered a good alternative protein source, as it contains about 35–57% crude protein with an essential amino acid profile similar to fishmeal and soybean (Veldkamp and Bosch 2015), more than 28% of lipids, and minerals such as calcium and phosphorus (Makkar et al. 2014, Wang and Shelomi 2017). Most of the studies also showed that HILM can be a suitable alternative protein source for poultry (Marono et al. 2017; Dalle Zotte et al. 2018; Cullere et al. 2019; Gariglió et al. 2019). However, the proportion replacement of soybean meal or fish meal needs further investigation to avoid the negative effects, further, its application on laying hens has rarely been reported. We postulated that the use of proportions of HILM in compound feed with basal diet would make it possible to fully exploit the growth potential of laying hens. Therefore, this study aimed to evaluate the effects of HILM on performance, egg quality, yolk fatty acid composition, egg amino acid composition, oxidative status, and immune response of laying hens. This study is anticipated to increase our knowledge about the potential of HILM as a protein feed replacement in laying hens, and provide novel and more detailed information on insect meal as a sustainable protein source rich in nutrients for poultry nutrition.

Materials and methods

All the birds and the experimental protocols in this study were approved by the Institutional Animal Care and Use Committee of Hunan Agricultural University, Hunan, China.

Table 1. Composition and nutrient levels of diets (air-dry basis).

| Items         | CON     | HILM-1  | HILM-3  | HILM-5  |
|---------------|---------|---------|---------|---------|
| Ingredientsa | (%)     |         |         |         |
| Corn          | 63.80   | 62.10   | 58.00   | 54.00   |
| Soybean meal  | 23.20   | 22.00   | 19.50   | 17.00   |
| Wheat bran    | 0.00    | 1.90    | 6.50    | 11.00   |
| Limestone     | 7.00    | 7.00    | 7.00    | 7.00    |
| HILMb         | 0.00    | 1.00    | 3.00    | 5.00    |
| Ca(HCO3)2     | 1.00    | 1.00    | 1.00    | 1.00    |
| Premixc       | 5.00    | 5.00    | 5.00    | 5.00    |
| Total         | 100.00  | 100.00  | 100.00  | 100.00  |
| Calculated composition |     |         |         |         |
| ME (MJ/kg)    | 10.97   | 11.00   | 11.00   | 11.02   |
| CP            | 15.81   | 15.80   | 15.81   | 15.81   |
| EE            | 2.74    | 3.09    | 3.80    | 4.51    |
| Ca            | 2.89    | 2.91    | 2.95    | 2.99    |
| Lys           | 0.89    | 0.90    | 0.90    | 0.91    |
| AP            | 0.54    | 0.56    | 0.58    | 0.61    |
| Met           | 0.45    | 0.48    | 0.50    | 0.52    |

aHILM, Hermetia illucens larvae meal; ME, metabolisable energy; CP, crude protein; EE, ether extract; Met, methionine; Lys, lysine; Ca, Calcium; AP, available phosphorus.
bCON, HILM-1, HILM-2, HILM-3 diets contained 0, 1%, 3%, 5% HILM, respectively.
cThe premix provided the following per kg of diets: vitamin A, 14 000 IU; vitamin B1, 1.3 mg; vitamin B2, 12 mg; vitamin B6, 16.5 mg; vitamin B12, 6 mg; vitamin B12, 0.03 mg; vitamin D3, 3 500 IU; vitamin E, 100 IU; vitamin K3, 4.25 mg; vitamin B1, 6 mg; vitamin B12, 0.03 mg; vitamin D3, 3 500 IU; vitamin E, 100 IU; vitamin K3, 4.25 mg; nicotinamide, 50 mg; D-biotin, 0.25 mg; folic acid, 2 mg; Cu, 12.5 mg; Fe, 70 mg; Mn, 110 mg; Zn, 85 mg; I, 1.25 mg; Se, 0.3 mg; Ca, 5 g; TP, 1.5 g; NaCl, 1.5 ~ 7.5 g; choline chloride, 400 mg.

Birds, diets, and experimental design

Briefly, a total of 432 healthy Xuefeng black-bone chickens with similar weight at 45 weeks of age were obtained from Hunan Yunfeifeng Agricultural Commercial Company (Hunan, China), and randomly assigned to four experimental groups with six replicates of 18 birds per replicate. A diet based on corn and soybean meal was formulated and served as control (C), while 1%, 3%, and 5% HILM inclusion as a partial replacement of soybean meal constituted the three experimental treatment groups (HILM-1, HILM-3, and HILM-5) (Table 1). The experimental diets were isonitrogenous and isoenergetic, differing in the use of proportions of HILM. The HILM was purchased from Hunan Yuxiong Ecological Agricultural Technology Co., Ltd (Yueyang, Hunan, China). Nutrient analyses of the HILM and the experimental diets were carried out in duplicate. The dry matter (DM) (GB/T 6435-2006), crude protein (CP) (GB/T 6432-1994), ether extract (EE) (GB/T 6433-2006), ash (GB/T 6438-2007), calcium (Ca) (GB/T 13885-2003), and phosphorus (P) (GB/T 6437-2002) contents of the selected samples were determined the standard laboratory procedures (Folch et al. 1957). Briefly, the moisture content was obtained by drying samples to a constant weight in an oven at 105 °C for 24 h. Crude protein was determined as total nitrogen (N) by using the Kjeldahl method (N × 6.25). Crude lipid was measured by ethyl ether extraction.
using a Soxtec system. Ash was obtained through combustion at 550°C for 5 h in the muffle furnace. The basal diet was formulated in accordance with the China Agricultural Standard (Wen et al. 2004) to meet the nutrient requirements of laying hens. The chemical composition of the experimental diets is reported in Table 1. Moreover, the chemical composition of the HILM was as follows: DM 95.02%, CP 36.94%, EE 42.27%, crude ash 10.76%, Ca 2.46%, P 0.74%, lysine 2.15%, methionine 0.60%.

This feeding experiment was conducted from March to June in 2019 at the original breeding farm of Hunan Yunfeifeng Agricultural Commercial Company. The hens were raised in a wire cage with 3 ladders, and 3 hens were raised in a cage (40 × 40 × 38 cm; length × width × height). The replicates were allotted equally into the middle cages to minimise the effects of the cage level. Hens were housed in an environmentally controlled room. During the experiment, the temperature and relative humidity in the room were 25.24°C and 67.31%, respectively. The hens were allowed a period of 1 week to adapt to the environment. Then, all hens were fed the assigned experimental diets for 56 days. The hens were fed twice a day (08:00 h and 15:00 h) and given ad libitum access to water throughout the experiment. The lighting regimen used was a 16-h light and 8-h darkness cycle.

Measurement of production performance and egg quality

Egg production and egg weight were recorded daily by replicate, and feed consumption was recorded weekly by replicate to calculate egg production, feed intake, feed conversion ratio (FCR). Egg quality was measured on four eggs collected randomly from each replicate at d 28 and d 56. The length and width of the egg, the width and height of yolk, and the albumen height were measured by using an electronic digital calliper (SH14100025, Shenhon, Shanghai, China). The egg-shape index was calculated by dividing egg length by egg width, and the yolk index was calculated by dividing yolk height by yolk width. Eggshell-breaking strength was measured by an egg force reader (EFR-01, Orka Food Technology Ltd, Israel). Haugh unit were measured by using an egg analyser (EA-01, Orka Food Technology Ltd, Ramat HaSharon, Israel). Eggshell thickness was measured by a digital micrometer (NFPN380, FHK, Japan) at three different locations (bottom, middle, and top of the egg) and then was calculated from the average shell thickness as described by Liu et al. (2017). Eggshell weight was measured by using electronic scales.

Yolk fatty acid composition

The fatty acid composition of the egg yolk was determined according to a previously described method (Li et al. 2015). Briefly, total lipids were extracted from the approximately 0.5 g separated egg yolk (lyophilized sample) using petroleum ether/anhydrous diethyl ether (1:1, v/v). Methyl esters of the lipids were prepared using saponification with a solution of KOH:methanol (4 mol:1 L). The organic layer was aspirated for fatty acid analysis using an Agilent 7890 N gas chromatography equipped with a flame ionisation detector (Agilent Technologies, Santa Clara, CA, USA) and a CP-Sil 88 fused silica open tube capillary column (100 m × 0.25 mm; Agilent Technologies, USA). The gas chromatograph temperature program was as follows: Initial temperature of 140°C for 5 min, temperature increase of 3°C/min to 220°C, 1 min temperature hold at 220°C, and then holding temperature at 220°C for additional 40 min. The injector and detector temperatures were maintained at 240°C and 260°C, respectively. Hydrogen was used as the carrier gas at a flow rate of 40 mL/min. Individual fatty acid peaks were identified by comparing their retention times with those of the standards (Cat#: 18919-1AMP; Sigma Chemicals, St. Louis, MO, USA). The results were expressed as grams per 100 g of total identified fatty acids.

Saturated fatty acid (SFA) total proportion was the weighted percentage sum of myristic (14:0), palmitic (16:0), margaric (17:0), and stearic (18:0) acids. Total proportions of monounsaturated fatty acids (MUFA) included myristoleic (14:1), palmitoleic (16:1c9), elaidic acid (C18:1t9), oleic acid (18:1c9), and eicosenoic acid (C20:1). Additionally, the polyunsaturated fatty acid (PUFA) total percent summed linoleic (18:2n6), γ-linolenic (18:3n6), α-linolenic (18:3n3), eicosadienoic (20:2), dihomo-γ-linolenic (20:3n6), arachidonic (20:4n6), and docosahexaenoic (22:6n3) acid.

Egg amino acid traits

The amino acid composition of the egg was determined according to a previously described method (Mori et al. 2020). Lyophilized egg sample (100 mg) was mixed with 10 mL of 6 mol/L hydrochloric acid solution. After vortexing, the samples were centrifuged at 1400g for 15 min using a table-top centrifuge, model 2410 (KUBOTA Corporation Co., Ltd., Japan).
The supernatant was collected using a 5-mL syringe (NIPRO Corporation, Japan) and filtered through a disposable cellulose acetate membrane filter unit with a 0.45-μm pore size (DISMIC-25CS; Advantec Toyo Kaisha, Ltd., Japan). After heating at 40°C for 60 min in a vacuum oven (VOS-201SD, Eyela, Japan), 20 mL of mixing solution (ethanol: DW: TEA = 2:2:1) was added to the tube and then mixed for 20 min using a microtube mixer MT-360 (Tomy Seiko Co. Ltd., Japan). The sample was heated at 40°C for 60 min in a vacuum to dry. After adding 20 mL of mixing solution (Ethanol: DW: TEA: PITC = 7:1:1:1) and mixing for 20 min, the sample was re-heated at 40°C for 60 min in a vacuum to dry. After preprocessing, the sample tube was maintained at −30°C until the sample was analysed.

Amino acids were analysed by HPLC (LC-2010CHT; Shimadzu Co. Ltd., Japan). Solutions of amino acid standards (types H and B), L-aspartic acid, and L-glutamic acid (FUJIFILM Wako Chemicals, Japan) were prepared following the same protocol used for sample preprocessing. The standard samples were analysed before every 30 samples. The absolute concentration of amino acids in egg was calculated from the peak ratio between sample and standard.

Blood sample collection

The immunisation program included vaccination against Newcastle disease (day 110, Intramuscular injection, 0.3 mL/per hen), and avian influenza disease (day 18, day 85, and day 210, Intramuscular injection, 0.5 mL/per hen) as described by Liu et al. (2019). At the end of the experiment, after 12 h of feed withdrawal, two hens were randomly selected from each replicate. Blood samples (about 6 mL/hen) were drawn from the wing vein using a disposable lancet, then immediately transferred into a heparinised tube. Blood samples were placed at room temperature for 1 h and then centrifuged at 2500 × g at 4°C for 10 min, stored in sterilised 1.5-mL Eppendorf tubes at −20°C for further analysis.

Plasma antioxidant indexes and immune indexes

Plasma samples were individually used to measure the activities of glutathione peroxidase (GSH-Px), total superoxide dismutase (T-SOD), total antioxidant capacity (T-AOC), catalase (CAT), and concentration of malondialdehyde (MDA) using an assay kit (A005; A001-1; A015-1; A007-2; A003-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) with a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, US) according to the instructions of the manufacturer. The levels of NDV-Ab and AIV-Ab were determined using enzyme-linked immunosorbent assay (ELISA) kits (E-80133; E-192022; R&D Systems, Minnesota, USA). Concentrations of immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), interferon-γ (IFN-γ); interleukin-2 (IL-2); interleukin-4 (IL-4) were determined using ELISA kits (CSB-E11232Ch; CSB-EQ027259Ch; CSB-E16200C; CSB-E08550Ch; CSB-E06755Ch; CSB-E06756Ch; Cusabio Biotech Co., Ltd, Wuhan, Hubei, China) with microplate reader according to the instructions of the manufacturer.

Statistical analyses

All the data were statistically analysed by one-way analysis of variance (ANOVA) and linear and quadratic
regression models using SPSS version 21.0 statistical software (SPSS Institute Inc., Chicago, Illinois), and the replicate was used as the experimental unit. Differences between treatments were determined by Tukey’s multiple comparisons test. Data were presented as means ± pooled standard error of the mean (SEM) and considered statistically significant at \( p \)-Value less than .05 (\( p < .05 \)).

### Results

#### Production performance

Results of production performance were shown in Table 2. Feed intake linearly and quadratically increased (\( p < .05 \)) at day 1 to 28 of the experiment, while FCR linearly and quadratically decreased (\( p < .05 \)) at day 29 to 56 of the experiment. In the overall period, HILM increased the egg weight (\( p < .05 \)), feed intake (\( p < .05 \)), and egg production (\( p < .05 \)), while decreased the FCR (\( p < .05 \)) in a linear or quadratic manner.

#### Egg quality

The effect of HILM on egg quality were listed in Table 3. As dietary supplementation of HILM increased, the eggshell weight quadratically increased (\( p < .05 \)), while the eggshell thickness, yolk index, and albumen height linearly decreased (\( p < .05 \)) at day 28 of the experiment. Dietary supplementation with HILM had no effect on egg quality at day 56 of the experiment.

#### Fatty acid composition

The effect of HILM on yolk fatty acid composition of laying hens were showed in Table 4. The levels of C14:0, C17:0, C18:2n6c, C20:2 linearly or quadratically increased (\( p < .05 \)) with a maximum observed for the HILM-3 group. While the levels of C16:0 and C16:1 linearly decreased (\( p < .05 \)) with increasing HILM levels. And the level of PUFA linearly increased (\( p < .05 \)) in response to increasing HILM levels.

#### Amino acid composition

Egg samples contained 17 amino acids: aspartic acid (Asp), glutamic acid (Glu), arginine (Arg), proline (Pro), alanine (Ala), serine (Ser), glycine (Gly), histidine (His), threonine (Thr), tyrosine (Tyr), cystine (Cys), valine (Val), methionine (Met), phenylalanine (Phe), leucine (Leu), isoleucine (Ile), lysine (Lys) (Table 5). The contents of Glu and Val linearly increased (\( p < .05 \)), the content of Met linearly and quadratically increased (\( p < .05 \)) with increasing HILM levels, with a maximum observed for the HILM-3 group.

#### Plasma antioxidant capacity

Results of antioxidative parameters in plasma were presented in Table 6. The activities of T-SOD and CAT linearly or quadratically increased (\( p < .05 \)) with increasing HILM levels, with a maximum observed for the HILM-3 group. While the content of MDA linearly decreased (\( p < .05 \)) in response to increasing HILM levels.
**Plasma immune indexes**

As showed in Table 7, the level of NDV-Ab in the plasma linearly increased ($p < .05$) with an increased HILM supplementation. While the concentration of IL-2 linearly decreased ($p < .05$), with a minimum observed for the HILM-3 group.

**Discussion**

Currently, insect meal displays a great potential for being a suitable ingredient in animal feeding, because of the high quality of protein (Premalatha et al. 2011). Insects can be an interesting alternative protein source in particular for poultry, considering that they are part of the ‘natural’ diet of chickens (Bovera et al. 2015).

### Table 4. Effect of HILM on yolk fatty acid composition of laying hens.

| Item | Dietary treatment | Pooled SEM | p-Value |
|------|------------------|------------|---------|
|      | CON | HILM-1 | HILM-3 | HILM-5 |           | ANOVA | Linear | Quadratic |
| C14:0 | 0.45<sup>b</sup> | 0.53<sup>b</sup> | 0.69<sup>a</sup> | 0.84<sup>a</sup> | 0.039 | .000 | .000 | .375 |
| C16:0 | 26.68 | 26.26 | 25.88 | 25.52 | 0.230 | .149 | .036 | .612 |
| C17:0 | 0.16<sup>b</sup> | 0.15<sup>b</sup> | 0.20<sup>a</sup> | 0.20<sup>a</sup> | 0.007 | .018 | .006 | .783 |
| C18:0 | 8.75 | 9.18 | 9.03 | 9.02 | 0.103 | .566 | .504 | .317 |
| SFA | 36.05 | 36.62 | 35.79 | 35.58 | 0.236 | .469 | .311 | .424 |
| C14:1 | 0.15 | 0.15 | 0.18 | 0.19 | 0.009 | .275 | .071 | .911 |
| C16:1 | 4.09 | 3.72 | 3.48 | 3.10 | 0.141 | .075 | .011 | .984 |
| C18:0 | 0.18<sup>b</sup> | 0.15<sup>b</sup> | 0.20<sup>a</sup> | 0.20<sup>a</sup> | 0.004 | .559 | .408 | .584 |
| C18:1<sup>o3</sup> | 42.32 | 42.06 | 41.62 | 42.18 | 0.391 | .941 | .822 | .633 |
| C20:1 | 0.25 | 0.24 | 0.26 | 0.25 | 0.006 | .855 | .567 | 1.000 |
| MUFA | 46.97 | 46.34 | 45.71 | 45.89 | 0.330 | .573 | .220 | .559 |
| C18:2<sup>o6</sup> | 11.98 | 12.14 | 13.46 | 13.60 | 0.309 | .118 | .026 | .986 |
| C18:3<sup>o9</sup> | 27.16 | 28.18 | 28.49 | 27.56 | 0.030 | .508 | .619 | .163 |
| C20:1<sup>o9</sup> | 16.89 | 17.30 | 17.73 | 17.20 | 0.023 | .565 | .479 | .270 |
| C20:4<sup>o6</sup> | 2.90 | 2.92 | 2.86 | 2.96 | 0.041 | .910 | .713 | .705 |
| PUFA | 16.94 | 17.04 | 18.46 | 18.51 | 0.312 | .114 | .028 | .965 |

<sup>a</sup>Data are means of six replicates per treatment with 2 hens per replicate;  
<sup>b</sup>HILM, *Hermetia illucens* larvae meal; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids;  
<sup>c</sup>CON, HILM-1, HILM-3, HILM-5 diets contained 0, 1%, 3%, 5% HILM, respectively;  
<sup>a-b</sup>Means within a row with different superscripts differ significantly ($p < .05$).

### Table 5. Effect of HILM on yolk amino acid composition of laying hens.

| Item | Dietary treatment | Pooled SEM | p-Value |
|------|------------------|------------|---------|
|      | CON | HILM-1 | HILM-3 | HILM-5 |           | ANOVA | Linear | Quadratic |
| NEAA | Aspartic Acid (Asp) | 46.79 | 48.16 | 48.44 | 47.96 | 0.545 | .752 | .469 | .428 |
|      | Glutamic Acid (Glu) | 56.93<sup>b</sup> | 58.00<sup>b</sup> | 61.40<sup>a</sup> | 60.23<sup>a</sup> | 0.626 | .024 | .009 | .264 |
|      | Arginine (Arg) | 27.16 | 28.18 | 28.49 | 27.56 | 0.030 | .508 | .619 | .163 |
|      | Proline (Pro) | 16.89 | 17.30 | 17.73 | 17.20 | 0.203 | .565 | .479 | .270 |
|      | Alanine (Ala) | 23.95 | 24.76 | 25.19 | 24.61 | 0.029 | .548 | .382 | .266 |
|      | Serine (Ser) | 33.25 | 34.03 | 34.95 | 33.59 | 0.388 | .472 | .585 | .192 |
|      | Glycine (Gly) | 14.14 | 14.56 | 15.03 | 14.53 | 0.186 | .433 | .342 | .230 |
| EAA | Histidine (His) | 10.33 | 10.72 | 10.85 | 10.43 | 0.142 | .562 | .756 | .180 |
|      | Threonine (Thr) | 20.83 | 21.51 | 21.98 | 21.33 | 0.245 | .449 | .388 | .195 |
|      | Tyrosine (Tyr) | 14.12 | 14.65 | 15.53 | 14.81 | 0.216 | .136 | .115 | .138 |
|      | Cystine (Cys) | 4.05 | 4.11 | 4.45 | 4.39 | 0.079 | .181 | .056 | .685 |
|      | Valine (Val) | 26.60<sup>b</sup> | 27.07<sup>ab</sup> | 29.17<sup>a</sup> | 27.82<sup>ab</sup> | 0.352 | .046 | .042 | .141 |
|      | Methionine (Met) | 7.58<sup>b</sup> | 8.65<sup>b</sup> | 9.96<sup>a</sup> | 8.41<sup>ab</sup> | 0.248 | .001 | .013 | .001 |
|      | Phenylalanine (Phe) | 22.82<sup>b</sup> | 22.77<sup>b</sup> | 25.05<sup>a</sup> | 23.46<sup>a</sup> | 0.323 | .039 | .088 | .172 |
|      | Leucine (Leu) | 37.50<sup>ab</sup> | 37.57<sup>ab</sup> | 40.62<sup>a</sup> | 38.48<sup>ab</sup> | 0.452 | .048 | .087 | .170 |
|      | Isoleucine (Ile) | 22.60 | 23.37 | 23.89 | 23.11 | 0.310 | .557 | .477 | .242 |
|      | Lysine (Lys) | 29.50 | 30.91 | 30.94 | 30.49 | 0.361 | .492 | .374 | .223 |
| Total | 416.31 | 430.26 | 440.35 | 427.32 | 5.177 | .464 | .369 | .215 |

<sup>a</sup>Data are means of six replicates per treatment with 2 hens per replicate;  
<sup>b</sup>HILM, *Hermetia illucens* larvae meal; NEAA, nonessential amino acid; EAA, essential amino acid;  
<sup>c</sup>CON, HILM-1, HILM-3, HILM-5 diets contained 0, 1%, 3%, 5% HILM, respectively;  
<sup>a-b</sup>Means within a row with different superscripts differ significantly ($p < .05$).
Many researchers have also evaluated the feasibility of supplementing poultry diets using insects such as the HILM, the maggot meal or the yellow mealworm (Premalatha et al. 2011; Marono et al. 2017).

In the present study, we fed laying hens with a basal diet supplemented with different levels of HILM. Laying hens showed satisfactory productive performance throughout the trial. Supplementation with 3% HILM or 5% HILM in the diet significantly increased the egg weight, feed intake, decreased FCR of laying hens during the whole experimental period. The current results were corroborated by Marono et al. (2017) and Bovera et al. (2018), who observed that fed with HILM as partial replacement and as a component of a complete diet of soybean meal improved egg mass, feed intake, egg weight in laying hens. The increased feed intake observed in the laying hens of present study can be attributed to the improvement of the diet palatability related to HILM (Bovera et al. 2015; Cullere et al. 2016). It is possible to assume that the improvement of production performance is related to the improvement of metabolism, immunity and intestinal health (Cutrignelli et al. 2018; Cai et al. 2018; Sypniewski et al. 2020). However, Schiavone et al. (2018) showed that 50% or 100% replacement of soybean oil with HILM in broiler chickens diets has no adverse effects on the daily feed intake or feed conversion ratio. This finding was in agreement with the findings reported by Gariglio et al. (2019) and Sypniewski et al. (2020) who reported no adverse effects of HILM substitution in terms of final body weight, daily body weight gain, daily feed intake and FCR in other poultry. Furthermore, Marono et al. (2017) reported that when used in total substitution of soybean meal, HILM negatively affected feed intake and thus production performance of hens. Biasato et al. (2017) found that increasing levels of dietary T. molitor meal inclusion in male broiler chickens may improve body weight and feed intake, but negatively affect feed efficiency and intestinal morphology. Agunbiade et al. (2007) showed that maggot meal could replace 50% of fishmeal protein (5% in diet) without adverse effects on egg production and shell strength but 100% of replacement decreased egg production of 50-week-old laying hens, thus suggesting that low levels may be more suitable. We assumed that the variation in the results of the aforementioned studies also can be ascribed to several factors, including the stage of insects, the dose of HILM, the age of the animals, etc.

### Table 6. Effect of HILM on plasma antioxidant capacity of laying hens

| Item | CON | HILM-1 | HILM-3 | HILM-5 | Pooled SEM | ANOVA | Linear | Quadratic |
|------|-----|--------|--------|--------|------------|-------|--------|----------|
| GSH-PX (U/mL) | 7005.02 | 7096.83 | 6037.35 | 6621.26 | 230.609 | .387 | .298 | .600 |
| T-SOD (U/mL) | 150.46<sup>a</sup> | 202.88<sup>a</sup> | 211.82<sup>a</sup> | 181.23<sup>ab</sup> | 7.888 | .017 | .102 | .005 |
| T-AOC (U/mL) | 4.46 | 4.48 | 5.16 | 4.15 | 0.292 | .690 | .928 | .405 |
| MDA (nmol/mL) | 7.86<sup>ab</sup> | 8.93<sup>a</sup> | 4.70<sup>ab</sup> | 4.17<sup>b</sup> | 0.667 | .024 | .006 | .486 |
| CAT (U/mL) | 3.76 | 4.52 | 5.98 | 5.87 | 0.431 | .197 | .047 | .605 |

<sup>A</sup>Data are means of two hens of six replicates per treatment.

<sup>B</sup>HILM, *Hermetia illucens* larvae meal; GSH-Px, glutathione peroxidase; T-SOD, total superoxide dismutase; T-AOC, total antioxidant capacity; MDA, malondialdehyde; CAT, catalase.

<sup>C</sup>CON, HILM-1, HILM-3, HILM-5 diets contained 0, 1%, 3%, 5% HILM, respectively.

<sup>a</sup>-<sup>b</sup>Means within a row with different superscripts differ significantly (p < .05).

### Table 7. Effects of HILM on plasma immune indexes of laying hens

| Item | CON | HILM-1 | HILM-2 | HILM-3 | Pooled SEM | ANOVA | Linear | Quadratic |
|------|-----|--------|--------|--------|------------|-------|--------|----------|
| IgG (g/L) | 4.53 | 4.56 | 4.41 | 4.53 | 0.035 | .461 | .586 | .523 |
| IgM (g/L) | 1.74 | 1.75 | 1.68 | 1.72 | 0.016 | .428 | .330 | .651 |
| IgA (g/L) | 2.32 | 2.33 | 2.23 | 2.23 | 0.030 | .397 | .149 | .802 |
| NDV-Ab (S/P) | 0.75<sup>a</sup> | 0.81 | 0.90 | 0.95 | 0.033 | .096 | .014 | .967 |
| AV-Ab (S/P) | 0.09<sup>b</sup> | 0.09<sup>ab</sup> | 1.07<sup>ab</sup> | 1.01<sup>ab</sup> | 0.010 | .044 | .149 | .116 |
| IFN-γ (pg/mL) | 44.97 | 47.77 | 37.07 | 44.53 | 2.058 | .353 | .504 | .575 |
| IL-2 (pg/mL) | 49.96<sup>ab</sup> | 265.03<sup>a</sup> | 178.17<sup>ab</sup> | 202.08<sup>ab</sup> | 12.125 | .028 | .017 | .827 |
| IL-4 (pg/mL) | 14.26 | 12.54 | 11.75 | 12.33 | 0.755 | .696 | .338 | .480 |

<sup>A</sup>Data are means of two hens of six replicates per treatment.

<sup>B</sup>HILM, *Hermetia illucens* larvae meal; IgG, immunoglobulin G; IgM, immunoglobulin M; IgA, immunoglobulin A; NDV-Ab, newcastle disease virus antibody; AV-Ab, avian influenza virus antibody; IFN-γ, interferon-γ; IL-2, interleukin-2; IL-4, interleukin-4.

<sup>C</sup>CON, HILM-1, HILM-3, HILM-5 diets contained 0, 1%, 3%, 5% HILM, respectively.

<sup>a</sup>-<sup>b</sup>Means within a row with different superscripts differ significantly (p < .05).
Regarding egg quality, the results of this study found that HILM in diet has no adverse effects on egg quality at day 56 of the experiment, while the eggshell thickness, yolk index, and albumen height at day 28 of the experiment significantly decreased. It appeared that HILM had a negative effect on the egg quality of laying hens in the early stage of the experiment, but it was improved in the later stage of the experiment. HILM contains chitin, a polysaccharide present in arthropod’s exoskeleton. Chitin is not digestible by monogastric animals and can negatively affect the protein digestibility (Bovera et al. 2015). A similar figure was given by Ruhnke et al. (2018) who did not observe variations of HILM as soybean meal substitute on the eggshell strength and Haugh unit in laying hens, while the eggshell thickness significantly decreased. These results contradict the findings of Secci et al. (2019), Mwaniki et al. (2018), and Star et al. (2020) who found that HILM had no adverse effect on shell weights, yolk diameter, and Haugh unit in laying hens. However, Mwaniki et al. (2020) showed that defatted HILM improved eggshell strength and eggshell thickness. Marono et al. (2017) found that feeding laying hens 17% black soldier fly larvae meal (BSFLM) increased circulating plasma Ca levels relative to the control, despite the two diets having similar Ca concentration. This suggested that improved calcium absorption and metabolism in HILM-fed birds could also have contributed to the stronger eggshells. Besides, some research found that HILM can improve the colour of yolk, which was related to be rich in β-carotene and lutein in HILM, pigments responsible for an orange-yellow colour (Secci et al. 2019; Mwaniki et al. 2020). Therefore, egg yolk colour is an important index that we need to focus on in the next experiments.

Most of the experiments published focussed on laying performance and egg quality, but rarely on fatty acid composition and amino acid traits of yolk. Our study showed that yolk of laying hens feed with HILM contained a high percentage of monounsaturated fatty acids (MUFA, up to 47%), followed by saturated fatty acids (SFA, up to 37%) and polyunsaturated fatty acids (PUFA, up to 19%). C18:1n9c was the most common fatty acid. These results are in line with findings in several earlier studies (Barroso et al. 2017; Liland et al. 2017; Spranghers et al. 2017; Meneguz et al. 2018). The dietary incorporation of HILM modified the fatty acid profile: the content of PUFA significantly increased with an increased HILM supplementation, which was attributable to the increasing proportions of C18:2n6c and C20:2 FA. Additionally, lauric acid (C12:0) was shown to lower fat deposition in tissues as it is oxidised to CO2 more rapidly than other long-chain FA (DeLany et al. 2000) and thus less available to be stored in tissues or to be elongated to 14:0 and 16:0 (Rioux et al. 2003; Dalle Zotte et al. 2018). However, a similar lowering effect on the lipid deposition related to feeding HILM to yolk of laying hens was not observed in the present study. C12:0 was present at high levels in all larvae, but in very limited amounts in the diets. This strongly indicates that it is synthesised by the larvae, as suggested by Spranghers et al. (2017). The elongation and desaturation of 18:2n-6 is conducted by the same enzymatic machinery, and thus changing the relative abundance of substrates is reflected in the abundance of LC-PUFA products (Secci et al. 2019). To our knowledge, this is the first time to explore the effect of insect meal on amino acids of egg in poultry. In the present study, we found that the Glu, Val, Met, Phe contents of egg significantly increased, with a maximum observed for the HILM-3 group. It is well known that most of the feedstuffs used in poultry nutrition do not contain a sufficient quantity of Met. And a dietary deficiency of Met leads to a significant decline in feed intake, and as a consequence, impairs growth and feed efficiency (Graber et al. 1971; Conde-Aguilera et al. 2016).

Oxidative stress occurs when there is a disproportion of ROS production and the volume of antioxidant systems to control their damaging effects (Monaghan et al. 2009). The first line of defense against ROS is represented by the SOD-CAT enzyme mechanism. Many researches showed that the SOD-CAT are important cellular antioxidant enzymes whose role in oxyradical reduction of superoxide anions into hydrogen peroxide and subsequent decomposition to oxygen and water (Nordberg and Arnér 2001; Fawole et al. 2016). The research of HILM on antioxidant capacity and immunity is few in poultry, but mainly in aquaculture. In the present study, the higher T-SOD and CAT activities observed in HILM-fed groups could be due to the accumulation of hydrogen peroxide (H2O2), thereby triggering the activity of catalase enzyme to decompose H2O2 and resulting in higher induction of CAT in HILM groups compared with the control. Our observation is in line with the opinion of Li et al. (2017) and Fawole et al. (2020) who found that CAT activities in fish increased when including HILM in their diets. Similarly, Xu et al. (2020) showed that increasing HILM dietary content significantly increased plasma CAT activity and decreased MDA content of the juvenile mirror carp. Besides, we also found that the plasma oxidative metabolites were affected by the dietary treatments.
and showed decreased values of MDA as a result of the dietary inclusion levels of HILM. The result was in consistent with the previous study of Gariglio et al. (2019), who reported that the content of MDA showed a linear decrease in plasma of Muscovy ducks fed increasing dietary levels of HILM. In the present study, dietary inclusion of 3% HILM meal markedly improved the level of NDV-Ab antibody, and decreased the level of IL-2 of laying hens. Insect were known to contain a significant amount of chitin in their exoskeleton. Therefore, the HILM could improve the antioxidant capacity of laying hens maybe because of the immunostimulating and anti-inflammatory effect of chitin (Khoushab and Yamabhai 2010). However, as studies on the effects of HILM on antioxidant capacity and immune response of broiler breeder are very little, further study is still needed to investigate its effect on the poultry.

**Conclusions**

In conclusion, the results demonstrate that supplementation of 3% HILM in broiler breeder could significantly improve egg weight and feed intake. Additionally, HILM can enhance the activity of T-SOD and the level of AIV-Ab in the plasma. Therefore, HILM is a suitable total substitute for soya bean meal in the diet of Xuefeng black-bone chicken.

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