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Effects of yellow mealworm larvae (*Tenebrio molitor*) inclusion in diets for female broiler chickens: implications for animal health and gut histology

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Abstract

The aim of the present study was to evaluate the animal performance, haematochemical parameters, intestinal morphology and histological features of broiler chickens fed diets including *Tenebrio molitor* (TM) larvae meal. A total of 160 female broiler chicks (Ross 708) at one-day of age were randomly allotted to four dietary treatments: a control (C) group and three TM groups, in which TM meal was included at 50, 100 and 150 g/kg, respectively. Each group consisted of five pens as replicates, with eight chicks per pen. After the evaluation of growth performance and haematochemical parameters, two birds per pen were slaughtered at 40 days and carcass traits were recorded. Morphometric investigations were performed on duodenum, jejunum and ileum and histopathological alterations were assessed for liver, spleen, thymus, bursa of Fabricius, kidney and heart. The live weight (LW) showed a linear (12 days, $P < 0.05$, maximum with TM15) and quadratic response (40 days, $P < 0.05$, maximum with TM5) to dietary TM meal inclusion. The average daily gain (ADG) showed a linear increase (1-12 days, $P < 0.05$, maximum with TM15) in response to TM meal utilization. A linear effect (1-12 and 12-25 days, $P < 0.01$ and $P < 0.05$, maximum with TM15 and TM5) was observed for the daily feed intake (DFI). The feed conversion ratio (FCR) showed a linear response to TM utilization in the period 12-25 days (P <

0.01, maximum with TM15). A quadratic effect ($P < 0.05$, maximum with TM5) was observed for the carcass weight. The abdominal fat weight and percentage showed a linear response to dietary TM meal inclusion ($P < 0.05$ and $P < 0.01$, maximum with TM15 and TM10). A quadratic increase ($P < 0.05$, maximum with TM10) was observed for the erythrocytes, while the albumin and GGT showed a linear and quadratic decrease ($P < 0.05$, minimum with TM10) in relation to TM utilization. Gut morphology and histopathological findings were not significantly influenced ($P > 0.05$) by dietary TM meal inclusion. The present study suggests that increasing levels of dietary TM meal inclusion in female broiler chickens diets may improve body weight and feed intake, but can partially worsen feed efficiency. However, positive effects on carcass traits and haematochemical parameters related to TM meal utilization are observed, along with no negative influence on gut morphology and histological findings.

Keywords

Poultry; *Tenebrio molitor*; insect meal; growth performance; histology; morphometry.

Introduction

World population is expected to increase by over a third, reaching over 9 billion people in 2050. This trend suggests that market demand for food will continue to grow. In particular, the demand for cereals and protein sources in both human food and animal feed is projected to have an exponential growth by 2050 (FAO, 2013). Consequently, the world supply of some conventional feedstuffs like soybean and maize will increasingly compete between humans and livestock. Therefore, the foremost gamble will be the identification of alternative sources of protein, energy and other nutrients for livestock, in order to avoid such a competition.

The potential of insects for becoming a standard ingredient in animal feeds has already been emphasized by several studies (Veldkamp et al., 2012; Van Huis, 2013; Henry et al., 2015), because of the high quality and quantity of protein (Makkar et al., 2014), the low competitiveness with human food (Ballitoc and Sun, 2013) and the reduction of the environmental impact (Oonincx and de Boer, 2012; Makkar et al., 2014; Sánchez-Muros et al., 2014). Currently, the considered most valuable insect species to be used in livestock feeds are *Hermetia illucens* L. (black soldier fly), *Musca domestica* L. (common house fly), *Tenebrio molitor* L. (yellow mealworm), *Bombyx mori* L. (silkworm) and several grasshoppers (Van Huis, 2013). In particular, *Tenebrio molitor* (TM) and *Hermetia illucens*

121 have recently been used in poultry (Biasato et al., 2016; Bovera
122 et al., 2016; Schiavone et al., 2017a) and fish (Belforti et al.,
123 2015; Gasco et al., 2016; Renna et al., 2017) feeding. Yellow
124 mealworms are already industrially produced as feed for pets
125 and zoo animals, such as birds, reptiles, small mammals,
126 amphibians and fish, and TM larvae are easily bred on dried
127 waste materials, being able to recycle them into high-quality
128 feed with less energy cost, land area utilization and footprints
129 (Makkar et al., 2014). The influence of TM-based diets on
130 growth performance (Bovera et al., 2015; Bovera et al., 2016;
131 Biasato et al., 2016) haematochemical profile (Bovera et al.,
132 2015; Biasato et al., 2016) and carcass traits (Ballitoc and Sun,
133 2013; Bovera et al., 2016; Bisato et al., 2016), has recently
134 been investigated. Gut morphology, which has been reported to
135 be widely affected by dietary modifications in broilers
136 (Laudadio et al., 2012; Gopinger et al., 2014; Qaisrani et al.,
137 2014), has also been evaluated in free-range chickens fed diets
138 with dietary TM larvae meal inclusion (Biasato et al., 2016).
139 Despite insect meals being considered suitable ingredients for
140 poultry feeding (Veldkamp et al., 2012; van Huis, 2013;
141 Makkar et al., 2014), the implications of their utilization on
142 poultry health and gut development are still very limited. The
143 aim of the present study was to evaluate the growth
144 performance, haematochemical parameters, carcass traits,

intestinal morphology and histological features of female broiler chickens fed diets including TM meal.

Materials and Methods

Birds and Husbandry

The present trial was performed in collaboration with a local poultry corporation named "O.R.A. Agricola S.r.l." sited in Cherasco (Cuneo, Italy). The experimental protocol was designed according to the guidelines of the current European and Italian laws on the care and use of experimental animals (European Directive 86 609/EEC, put into law in Italy with D.L. 116/92). Furthermore, the experimental protocol was approved by the Ethical Committee of the Department of Veterinary Sciences of the University of Turin (Italy). A poultry house of 14 m wide × 141 m long × 4.7 m high, equipped with waterproof floor and wall, completely covered by tiles and provided with automatic ventilation system was used.

A total of 160 female broiler chicks (Ross 708) at one-day of age were randomly allotted to 4 dietary treatments, each consisting of 5 pens as replicates with 8 chicks per pen. Each pen was 1.20 m wide × 1.20 m long and was equipped with a feeder occupying a surface of almost 1800 cm^2, three nipple drinkers and rice hulls as litter. During the first three weeks, the animals were heated by infrared lamps to maintain the suitable

temperature according to standard breeding practices (Aviagen, 2014). Lighting schedule was 23 hours light : 1 hour darkness until day 3 and 18 h light : 6 hours darkness until slaughter age. At hatching, all chicks received vaccination against Newcastle disease, Gumboro disease, infectious bronchitis and coccidiosis. Vaccine recalls were performed on day 9 for infectious bronchitis and on day 18 for Gumboro and Newcastle diseases.

Diets

A basal diet based on corn meal, corn gluten meal and soybean meal was formulated and served as control (C) group, while 50, 100 and 150 g/kg full-fat TM larvae meal (Gaobeidian Shannong Biology CO., LTD, Gaobeidian, Hebei province, China) inclusion as a partial replacement of soybean meal, corn gluten meal and soybean oil constituted the three experimental treatment groups (TM5, TM10 and TM15) (Table 1). TM meal nutritive composition and energy content were the following: dry matter, 939.0 (g/kg as fed); organic matter, 912.0 (g/kg as fed); crude protein (CP), 519.0 (g/kg as fed); ether extract (EE), 236.0 (g/kg as fed); 117.0 (g/kg as fed); neutral detergent fibre (NDF); 79.5 (g/kg as fed); acid detergent fibre (ADF); DL-methionine, 10.1 (g/kg as fed); L-lysine, 35.9 (g/kg as fed); gross energy, 24.4; apparent metabolizable energy (AMEn), 16.02 (MJ/kg DM). Three different diets were used per each

dietary treatments during the three phases of growth: a starter diet (days 1 to 12), a grower diet (days 12 to 25) and a finisher diet (day 25 to 40). For each phase, the experimental diets were isonitrogenous and isoenergetic and were formulated using the AMEn values for TM measured *in vivo* for broiler chickens (De Marco et al., 2015). Diets met or exceeded NRC (1994) requirements and were adjusted according to Aviagen (2014) broiler nutrition specifications. Feed and water were provided *ad libitum*.

Chemical analysis

The diets were ground to pass through a 0.5-mm sieve and stored in airtight plastic containers for DM (method number 943.01), ash (method number 924.05), CP (method number 954.01), EE (method number 920.39), NDF (method number 2002.04) and ADF (method number 973.18) determination (AOAC, 2004).

Growth Performances

Health status and mortality were daily monitored during the whole experimental period. Live weight (LW) of the animals was recorded at an individual level at the beginning of the trial, at day 12, 25 and 40. Average daily gain (ADG) and average daily feed intake (DFI) were recorded at an individual and a pen level, respectively, at the end of each growth period. Feed

conversion ratio (FCR) was determined for each growth period and for the overall experimental period. All measurements were made on the pen basis using a high precision electronic scale (Sartorius – Signum®).

Slaughtering procedure and recordings

At day 40, all birds were individually weighed and 10 broilers/diet (2 birds/pen) were chosen on the basis of pen average LW and identified with a shank ring.

All selected animals were slaughtered in a commercial abattoir. The plucked and eviscerated carcasses were obtained and the head, neck, feet and abdominal fat were removed to obtain carcass-for-grilling. The weights of liver, spleen, gizzard and abdominal fat were immediately recorded. All slaughtered carcasses were stocked in a cooling chamber (0-4 °C) for 24 h. Weights of carcass-for-grilling, breast and thighs were successively recorded. Carcass-for-grilling, breast, thigh and organs weights were also expressed as percentage of LW.

Collected feet were examined macroscopically using the Swedish FPD scoring system (Ekstrand et al., 1997). According to this system, 0 = no lesion, slight discoloration of the skin or healed lesion; 1= mild lesion, superficial discoloration of the skin and hyperkeratosis; 2 = severe lesion, affected epidermis, blood scabs, hemorrhage and severe swelling of the skin.

Haematological and serum parameters

At slaughter, blood samples were collected from the identified broilers: 2.5 mL was placed in an EDTA tube and 2.5 mL in a serum-separating tube. A blood smear was prepared, using one glass slide for each bird, from a drop of blood without anticoagulant. The smears were stained using May-Grünwald and Giemsa stains (Campbell, 1995). The total red and white blood cell counts were determined in an improved Neubauer haemocytometer on blood samples previously treated with a 1:200 Natt-Herrick solution. One hundred leukocytes, including granular (heterophils, eosinophils and basophils) and non-granular (lymphocytes and monocytes) leukocytes, were counted on the slide and the heterophils to lymphocytes (H/L) ratio was calculated. The tubes without anticoagulant were left to clot in a standing position at room temperature for approximately two hours to obtain serum. The serum was separated by means of centrifugation at 700 × g for 15 minutes and frozen at -80°C until analysis. The total proteins were quantified by means of the "biuret method" (Bio Group Medical System kit; Bio Group Medical System, Talamello (RN), Italy); the electrophoretic pattern of the serum was obtained 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,

glucose, phosphorus, magnesium, iron, uric acid and creatinine serum concentrations were measured by means of enzymatic methods in a clinical chemistry analyzer (Screen Master Touch, Hospitex diagnostics Srl., Firenze, Italy).

Histomorphological investigations

The slaughtered animals were submitted to anatomopathological investigations. Intestinal segment samples (approximately 5 cm in length) of duodenum, jejunum, ileum and caecum were excised from each bird and flushed with 0.9% saline to remove all the content. The collected segments of intestine were the loop of the duodenum, the tract before Meckel's diverticulum (jejunum), the tract before the ileocolic junction (ileum) and the apex of the caeca (caecum). Gut segments were fixed in Carnoy's solutions for morphometric analysis. Tissues were routinely embedded in paraffin wax blocks, sectioned at 5 µm thickness, mounted on glass slides and stained with Haematoxylin & Eosin (HE). The evaluated morphometric indices were Vh (from the tip of the villus to the crypt), Cd (from the base of the villus to the submucosa) and the Vh/Cd ratio (Laudadio et al., 2012). Morphometric analyses were performed on 10 well-oriented and intact villi and 10 crypts chosen from duodenum, jejunum and ileum (Qaisrani et al., 2014). Samples of liver, spleen, thymus, bursa of Fabricius, kidney and heart were also collected from each animal and

295 fixed in 10% buffered formalin solution for histopathological
296 examination. Tissues were processed in the same way as gut
297 and the following histopathological alterations were evaluated:
298 white pulp hyperplasia and depletion in spleen, cortical
299 depletion in thymus, follicular depletion and intrafollicular
300 cysts in bursa of Fabricius and lymphoid tissue activation in
301 liver (Biasato et al., 2016). Heart and kidney were assessed for
302 inflammatory and degenerative diseases. The observed
303 histopathological findings were evaluated using a
304 semiquantitative scoring system as previously assessed by
305 Biasato et al. (2016): absent/minimal (score = 0), mild (score =
306 1) and severe (score = 2).
307
308 ***Statistical Analysis***
309 IBM SPSS Statistics V20.0.0 software was used to perform
310 statistical analysis. Shapiro-Wilk's test established normality or
311 non-normality of distribution. The experimental unit was the
312 pen for growth performance, haematochemical parameters and
313 carcass traits and bird for histomorphological findings. Data
314 collected for growth performance, blood parameters and
315 carcass traits were tested by one-way ANOVA, evaluating the
316 effect of dietary TM inclusion by polynomial contrasts. χ^2 test
317 was performed to evaluate the association between the
318 mortality rate and the dietary treatments. Intestinal
319 morphometric indices were analyzed by fitting a general linear

model (GLM). The GLM 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). The interactions between the levels of the fixed factors were evaluated by pairwise comparisons. Statistical analysis was performed by procedure "General Linear Models > Univariate". Histopathological and FPD scores were analyzed by Kruskal-Wallis test (post-hoc test: Dunn's Multiple Comparison test). P values < 0.05 were considered statistically significant. The results were expressed as mean and pooled standard error of the mean (SEM).

Results

Growth performance

No clinical signs were observed and the birds remained healthy during the whole experimental period. The mortality rates of C (2.5%), TM5 (2.5%), TM10 (0%) and TM15 (2.5%) groups were not significantly different among treatments ($P > 0.05$). Growth performance of the broiler chickens are summarized in Table 2. At 12 days of age, the LW increased linearly with increasing TM meal levels ($P < 0.05$) and the linear response increased to a maximum corresponding to the inclusion of 150 g/kg of TM meal. At 25 days of age, no significant effects related to TM meal utilization were observed. At 40 days of age, the LW showed quadratic response to increasing TM meal

345 levels ($P < 0.05$), with a maximum corresponding to the
346 inclusion of 50 g/kg of TM meal. In the period from 1-12 days
347 of age, the ADG increased linearly with increasing TM meal
348 levels ($P < 0.05$) and the linear response increased to a
349 maximum corresponding to the inclusion of 150 g/kg of TM
350 meal. On the contrary, the ADG showed no differences ($P >$
351 0.05) in the periods from 12 to 25 and 25 to 40 days of age In
352 the period from 1 to 12 days of age, the response of DFI to the
353 effect of TM meal inclusion was statistically significant ($P <$
354 0.01). In particular, the linear response increased to a maximum
355 corresponding to the inclusion of 150 g/kg of TM meal. In the
356 period from 12 to 25 days of age, the DFI increased linearly
357 with increasing TM meal levels ($P < 0.05$) and the linear
358 response increased to a maximum corresponding to the
359 inclusion of 50 g/kg of TM meal. On the contrary, the DFI
360 showed no differences ($P > 0.05$) in the period from 25 to 40
361 days of age. In the periods from 1 to 12, 25 to 40 and 1 to 40
362 days of age, the FCR was similar among the dietary treatments
363 ($P > 0.05$). Differently, the FCR showed linear response to
364 increasing TM meal levels in the period from 12 to 25 ($P <$
365 0.01), with a maximum corresponding to the inclusion of 150
366 g/kg of TM meal.
367
368 ***Slaughtering performance and footpad dermatitis (FPD)***
369 ***score***

Table 3 summarizes the slaughtering performance of the broiler chickens. The carcass weight increased quadratically with increasing TM meal levels ($P < 0.05$) and the quadratic response increased to a maximum corresponding to the inclusion of 50 g/kg of TM meal. The abdominal fat weight showed linear response to increasing TM meal levels ($P < 0.05$), with a maximum corresponding to the inclusion of 150 g/kg of TM meal. Similarly, the abdominal fat percentage increased linearly with increasing TM meal levels ($P < 0.01$) and the linear response increased to a maximum corresponding to the inclusion of 100 g/kg of TM meal. On the contrary, no significant effects related to TM meal utilization were observed for the other carcass traits ($P > 0.05$). FPD scores (C: 0.40; TM5: 0.20; TM10: 0.20; TM15: 0.00) were also not influenced by dietary TM meal inclusion ($P > 0.05$).

Haematological and serum parameters

Haematological and serum biochemical traits of the broiler chickens are summarized in Table 4. The erythrocytes increased quadratically with increasing TM meal levels ($P < 0.05$) and the quadratic response increased to a maximum corresponding to the inclusion of 100 g/kg of TM meal. The albumin showed linear response to increasing TM meal levels ($P < 0.05$), with a minimum corresponding to the inclusion of 100 g/kg of TM meal. Similarly, the GGT decreased

quadratically with increasing TM meal levels ($P < 0.05$) and the quadratic response decreased to a minimum corresponding to the inclusion of 100 g/kg of TM meal. On the contrary, no significant effects related to TM meal utilization were observed for the other blood and serum parameters ($P > 0.05$).

Histomorphological investigations

The effects of the diet, intestinal segment and interaction between diet and intestinal segment on gut morphometric indices of the broiler chickens are shown in Tables 5 and 6. There was no influence of diet or interaction between diet and intestinal segment ($P > 0.05$) on the gut morphometric indices. On the contrary, Vh, Cd and Vh/Cd depended on intestinal segment ($P < 0.001$, $P = 0.001$ and $P < 0.001$, respectively) (Table 5). In particular, the duodenum showed higher Vh ($P < 0.001$) than the jejunum and ileum. Furthermore, higher Cd ($P < 0.001$) was found in the duodenum and jejunum than the ileum. Similarly, the duodenum and jejunum showed higher Vh/Cd ($P < 0.001$) than the ileum (Table 6).

Histopathological alterations were observed in all the dietary treatments and developed in spleen, thymus, bursa of Fabricius and liver, while heart and kidney showed no significant findings. Spleen (C = 1.50 ± 0.22; TM5 = 1.20 ± 0.25; TM10 = 1.10 ± 0.31; TM15 = 1.10 ± 0.28), thymus (C = 0.10 ± 0.10; TM5 = 0.30 ± 0.15; TM10 and TM15 = 0.10 ± 0.10), bursa of

Fabricius (C = 1.90 ± 0.10; TM5 = 1.50 ± 0.27; TM10 = 1.80 ± 0.20; TM15 = 1.70 ± 0.21) and liver (C = 0.90 ± 0.23; TM5 = 1.10 ± 0.23; TM10 = 0.90 ± 0.23; TM15 = 0.30 ± 0.15) scores were not influenced by dietary TM meal inclusion. Spleen showed moderate (C = 30% of the broilers; TM5 = 40%; TM10 = 10%; TM15 = 30%) to severe (C = 60%; TM5 = 40%; TM10 = 50%; TM15 = 40%) white pulp depletion or hyperplasia. The 10% I, 20% (TM5), 40% (TM10) and 30% (TM15) of the animals had normal spleen (Fig. 1A-B). In thymus, moderate cortical depletion was found in all the groups (C = 10% of the broilers; TM5 = 30%; TM10 and TM15 = 10%). A normal thymus was observed in 90% I, 70% (TM5) and 90% (TM10 and TM15) of the animals. Bursa of Fabricius showed moderate (C and TM5 = 10% of the broilers; TM10 = 0%; TM15 = 10%) to severe (C = 80%; TM5 = 70%; TM10 = 90%; TM15 = 80%) follicular depletion. The 0% I, 20% (TM5), 10% (TM10) and 10% (TM15) of the animals had normal bursa of Fabricius (Fig. 1C-D). In liver, moderate (C, TM5 and TM10 = 50% of the broilers; TM15 = 30%) to severe (C = 20%; TM5 = 30%; TM10 = 20%; TM15 = 0%) perivascular lymphoid tissue activation. A normal liver was observed in 30% I, 20% (TM5), 30% (TM10) and 70% (TM15) of the animals.

Discussion

Growth performances

Growth performances of the broiler chickens of the present study were consistent with the reference values recorded in the commercial farm in which the trial was conducted.

The body weight, weight gain and feed intake of the birds in the present trial improved with increasing levels of TM meal inclusion, but the feed efficiency resulted partially impaired. Little information on the influence of dietary TM meal inclusion in broiler chickens is currently available. Ramos-Eldoury et al. (2002) and Biasato et al. (2016) did not show any effects for the growth performance in fast-growing and intermediate-growing chickens, respectively, fed diets in which the TM inclusion level ranged from 50 to 100 g/kg. Differently, Ballitoc and Sun (2013) and Bovera et al. (2015) observed improved growth performance in fast-growing chickens fed diets with low (from 5 to 100 g/kg) or high (296 g/kg) TM inclusion levels, respectively. Similar findings were obtained by Loponte et al. (2017) in partridges fed diets in which the TM inclusion level ranged from 250 to 500 g/kg. Recently, Islam and Yang (2017) also found a positive effect of a mealworm-based probiotic on broiler growth performance. Some authors also explored the possibility to use other insect meals in poultry feeding. Adeniji (2007) and Hwangbo et al. (2009) studied the effects of housefly-maggots as feed supplement in the diet of broiler chickens: the first found no differences for growth performance with inclusion levels ranging from 55 to 220 g/kg,

while the latter (inclusion rate: 50-200 g/kg) observed a linear increase in LW gain. Ijaiya and Eko (2009) evaluated the effects of replacing dietary fishmeal with silkworm meal (inclusion rate: 22-93 g/kg) on growth performance of broiler chickens, finding no differences related to insect meal utilization. Also Oyegoke et al. (2006) and Wang et al. (2005) observed no adverse effects on growth performance of broiler chickens fed diets with *Cirina forda* (inclusion rate: 20-40 g/kg) and *Gryllus testaceus* (inclusion rate: 50-150 k/kg), respectively. Cullere et al. (2016) recently studied the influence of the inclusion (from 100 to 150 g/kg) of *Hermetia illucens* meal in quail diet, finding no differences for growth performances. Schiavone et al. (2017b) also evaluated the effects of replacing soybean oil with *Hermetia illucens* meal (inclusion rate: 500-1000 g/kg) on growth performance of broiler chickens, finding no differences related to insect meal utilization. The wide variability of the results obtained in the previous studies may be related to the nutritive value of the insect meal used, which can be influenced by the species, the insect life stage (adult, larva or pupa) and the insect rearing substrate (Sànchez-Muros et al., 2014).

The improvement of feed intake observed in the birds fed TM diets of the present trial was considered suggestive of increased feed palatability in relation to the addition of yellow mealworms, since insects are naturally consumed by wild birds

and free-range poultry (Zuidhof et al., 2003). In particular, the increased DFI observed in the starter period, which was accompanied by increased LW and ADG and unaffected FCR, was quite relevant. Indeed, starter period (from hatch to 10 days) is considered the most important in broiler production, since growth and development take place at an incredible rate during it. In this period, the chicks' weight quadruples, thus influencing the following growth rate (Aviagen, 2014). On the contrary, the increased DFI observed in the growing period was accompanied by unaffected LW and ADG and subsequently impaired FCR, thus representing a negative effect related to TM meal utilization. De Marco et al. (2015) speculated that the chitin contained in the exoskeleton of the TM meal may negatively influence the apparent digestibility coefficient of the total tract of nutrients. Furthermore, Ravindran and Blair (1993) pointed out that the chitin of insects is difficult to digest by domestic poultry. As suggested by Rumpbold and Schlüter (2013), the partial chitin removal through high pressure processing could improve the use of insects as feeding ingredient thanks to disruption of the link between some chitin-bound proteins. However, the limited number of birds included in the current trial could have influenced the data interpretation. The results obtained need to be confirmed on a larger number of animals.

Slaughtering performance and footpad dermatitis (FPD) score

The majority of the carcass traits of the broilers in the present trial were not influenced by dietary TM meal inclusion, as previously observed by Bovera et al. (2016) and Biasato et al. (2016). Similar findings were obtained by Cullere et al. (2016) and Schiavone et al. (2017) in broiler quails and chickens fed diets with *Hermetia illucens* meal and fat, respectively. However, the carcass weight, abdominal fat weight and abdominal fat percentage increased with increasing levels of TM meal utilization. Loponte et al. (2017) also observed improved carcass weights when TM and *Hermetia illucens* meals were included in the diets of partridges. As already suggested by them, the differences in the eviscerated carcass weights can be partially explained by the increased final LW of the birds. Similar findings in terms of improved eviscerated carcass weights were obtained by Khatun et al. (2003), Hwangbo et al. (2009) and Ballitoc and Sun (2013), who also observed improved slaughter, dressed carcass, breast muscle and thigh muscle weights and dressing percentage in broilers fed diets with different insect meals inclusion. The differences in the abdominal fat weight and abdominal fat percentage observed in the birds of the present study are also in agreement with Ballitoc and Sun (2013) and suggests that yellow

544 mealworm utilization may improve fat mass in broiler chickens
545 (USDA, 2011).
546 The majority of FPD scores obtained in the present trial was
547 zero and no differences were found in relation to TM meal
548 utilization. This is a positive result, since a low prevalence and
549 severity of FPD is highly desirable as far as health of birds and
550 product quality are concerned (Meluzzi et al., 2008).
551
552 ***Haematological and serum parameters***
553 All the blood parameters obtained in the present trial fell within
554 the physiological ranges (Lumej, 2008), thus suggesting that
555 TM meal utilization does not affect health status of the animals.
556 In particular the H/L ratio, that is commonly used as indicator
557 of stress in poultry (De Marco et al., 2013; Salamano et al.,
558 2010), was not affected by dietary TM meal inclusion. As
559 already observed by Bovera et al. (2015) and Biasato et al.
560 (2016), the majority of the haematochemical and serum
561 biochemical traits were not affected by yellow mealworm
562 inclusion in the birds of the present study. Similar findings
563 were obtained by Schiavone et al. (2017b) in broilers fed diets
564 with *Hermetia illucens* fat. However, the erythrocytes increased
565 and albumin and GGT decreased with increasing levels of TM
566 meal utilization Loponte et al. (2017) also observed lower
567 albumin when TM and *Hermetia illucens* meals were included
568 in the diets of partridges. Interestingly, this finding was

accompanied by the increase of albumin/globulin ratio, which was also reported by Bovera et al. (2015) and ascribed to the properties of chitin contained in insect meal. High globulin concentrations and low albumin/globulin ratios generally indicate better disease resistance and immune response of birds (Griminger and Scanes, 1986). Another interesting finding is represented by the decrease of GGT serum levels in TM animals. Indeed, a high GGT concentration in birds is used as an indicator of liver disease and bile flow disorders (Ognik and Krauze, 2016). Therefore, GGT reduction can be considered a positive effect related to TM meal utilization.

Histomorphological investigations

Dietary TM meal inclusion did not influence the gut morphology of the birds of the present study, as already observed by Biasato et al. (2016). Morphometric measurements of Vh and Cd are generally used to assess intestinal development (Franco et al., 2006), since represent useful indicators of gut proliferative and absorptive compartments (Lenhardt and Mozes, 2003). The Vh/Cd ratio is also evaluated, because it gives an indication of the likely maturity and functional capacity of the enterocytes (Hampson, 1986). As previously reported (Uni et al., 1999; Iji et al., 2001; Biasato et al., 2016), the present study confirms that both duodenum and jejunum show a greater morphological development compared

with the ileum. Indeed, the duodenum is the intestinal tract with the fastest cell renewal, and is also the first segment of the small intestine to receive physical, chemical and hormonal stimuli provoked by diet (Macari, 1998). Furthermore, the jejunum is an important site for nutrient digestion (Iji et al., 2001). Therefore, dietary TM inclusion preserves a proximodistal decreasing gradient of the morphometric indexes from the duodenum to the ileum, thus suggesting the maintenance of the physiological gut development.

The broiler chickens of the present trial showed different degrees of lymphoid system activation, with no differences related to dietary TM meal inclusion. This result could be related to the stress occurrence in modern poultry rearing operations. Stress can be caused by a variety factors, physiological (rapid growth rate) and social (overcrowding) ones included (Liles et al., 2015). However, a great deal of individual bird variability in some immunological measures (i.e., stimulation index, heterophils to lymphocyte ratio and lymphocyte blastogenesis) may also be considered (Talebi et al., 1995).

Conclusion

In conclusion, the present study suggests that increasing levels of dietary TM meal inclusion in female broiler chickens diets may improve body weight, weight gain and feed intake, but can

partially worsen feed efficiency. However, positive effects on carcass traits and haematochemical parameters are observed, along with no negative influence on gut morphology and histological findings. These results confirm previous data concerning the safety of TM utilization in poultry feed, even if legislative issues are still needed to allow insect meal to be used as transformed animal protein to feed monogastric farm animals.

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860 **Table 1.** Ingredients (g/kg as fed), apparent metabolizable energy (MJ/kg DM) and nutrient composition (%) of the

861 experimental diets.

Ingredients	First Period (days 1 to 12)				Second period (days 12 to 25)				Third period (day 25 to slaughter)			
	Control	TM5	TM10	TM15	Control	TM5	TM10	TM15	Control	TM5	TM10	TM15
Corn meal	483.2	482.7	488.5	496.6	523.8	535.9	549.3	566.8	566.6	572.2	585.7	605.4
Soybean meal	345.0	333.8	304.0	262.0	317.0	294.0	254.1	203.9	275.5	259.0	219.0	164.0
TM meal	0.0	50.0	100.0	150.0	0.0	50.0	100.0	150.0	0.0	50.0	100.0	150.0
Corn gluten meal	75.5	42.0	23.0	14.5	58.3	24.0	8.0	0.0	56.0	21.0	5.0	0.0
Soybean oil	54.0	50.3	43.5	34.8	64.9	59.9	51.7	41.6	68.9	64.9	56.7	45.8
Dicalcium phosphate	11.0	12.0	13.0	15.5	8.4	9.0	10.5	12.9	7.0	8.0	9.5	12.0
Calcium carbonate	17.5	16.5	16.0	15.0	15.0	15.0	14.5	13.0	14.5	14.0	13.5	12.2
Sodium chloride	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3
Sodium bicarbonate	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
DL-methionine	0.8	0.9	0.9	0.8	0.8	0.9	0.9	0.8	0.4	0.6	0.6	0.5
L-lysine	3.1	1.9	1.2	0.9	2.0	1.3	0.9	0.8	1.3	0.4	0.0	0.0
Threonine	0.1	0.1	0.1	0.1	0.0	0.2	0.3	0.4	0.0	0.1	0.2	0.3
Trace mineral-vitamin premix[1]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Coline	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
3-phytase	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Total	100	100	100	100	100	100	100	100	100	100	100	100
AMEn[2] (MJ/kg)	12.89	12.89	12.89	12.89	13.28	13.28	13.28	13.28	13.54	13.54	13.54	13.54
Nutrient composition (%)												
DM	86.6	86.6	86.7	86.6	86.7	86.8	86.6	86.8	86.8	86.7	86.7	86.8
CP	23.5	23.5	23.6	23.8	21.3	21.1	21.1	21.1	19.6	19.6	19.6	19.6
EE	7.9	8.3	9.0	9.6	9.0	9.2	9.8	10.3	9.5	9.7	10.4	10.8
NDF	9.4	9.8	10.0	10.1	9.4	9.8	10.0	10.1	9.4	9.7	9.9	10.1
ADF	3.8	4.1	4.3	4.4	3.7	3.9	4.1	4.1	3.5	3.8	3.9	4.0

Nutrient composition (%)[2]												
Calcium	1.1	1.1	1.1	1.1	0.9	0.9	0.9	0.9	0.8	0.8	0.8	0.8
Available phosphorus	0.6	0.6	0.5	0.6	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Digestible methionine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.4
Digestible lysine	1.4	1.4	1.4	1.4	1.3	1.3	1.3	1.3	1.1	1.1	1.1	1.1
Digestible threonine	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.8	0.8	0.8	0.8

862 [1]Mineral-vitamin premix (Final B Prisma, IZA SRL), given values are supplied per kg of diet: 2.500.000 IU of vitamin A;
863 1.000.000 IU of vitamin D3; 7.000 IU of vitamin E; 700 mg of vitamin K; 400 mg of vitamin B1; 800 mg of vitamin B2; 400
864 mg of vitamin B6; 4 mg of vitamin B12; 30 mg of biotin; 3.111 mg of Ca pantothenate acid; 100 mg of folic acid; 15.000 mg
865 of vitamin C; 5.600 mg of vitamin B3; 10.500 mg of Zn, 10.920 mg of Fe; 9.960 mg of Mn; 3.850 mg of Cu; 137 mg of I; 70
866 mg of Se.

867 [2]Calculated according to INRA 2004 and De Marco et al. (2015).

868 TM, *Tenebrio molitor*; AME, apparent metabolizable energy; DM, dry matter; CP, crude protein; EE, ether extract; NDF,
869 neuter detergent fiber; ADF, acid detergent fiber.

870

871 **Table 2.** Effect of the dietary TM larvae meal inclusion on the growth performance of the female broiler chickens.

		Dietary treatments[1]					P[3]	
Variable[2]	**Age**	**C**	**TM5**	**TM10**	**TM15**	**SEM**	**Linear**	**Quadratic**
LW (g)	12 d	303.15	338.25	339.15	351.83	6.78	0.013	0.352
	25 d	1174.90	1234.77	1183.35	1179.79	14.14	0.775	0.280
	40 d	2078.46	2309.97	2115.91	2084.22	30.51	0.408	0.012
ADG (g)	1-12 d	23.61	26.80	26.89	28.04	0.61	0.012	0.350
	12-25 d	67.06	68.96	64.94	63.69	1.17	0.198	0.512
	25-40 d	60.24	71.68	62.17	60.29	1.91	0.552	0.071
DFI (g)	1-12 d	25.40	28.80	30.80	32.40	0.95	0.006	0.583
	12-25 d	95.00	117.00	109.40	116.60	2.95	0.014	0.129
	25-40 d	184.41	220.23	209.49	225.28	6.85	0.731	0.741
FCR (g/g)	1-12 d	1.08	1.07	1.14	1.15	0.03	0.336	0.925
	12-25 d	1.42	1.74	1.68	1.86	0.05	0.001	0.325
	25-40 d	2.40	2.16	2.13	2.31	0.11	0.775	0.404
	1-40 d	1.78	1.84	1.81	1.95	0.05	0.342	0.730

872 [1]Each mean represents 5 replicates with 8 chicks/replicate (n = 40/treatment).

873 [1]Four dietary treatments: C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level

874 of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*.

875 [2]LW, live weight; DFI, daily feed intake; ADG, average daily gain; FCR, feed conversion ratio; n, number of pens.

876 [3]Statistical significance: $P < 0.05$.

877

878 **Table 3.** Effect of the dietary TM larvae meal inclusion on the carcass traits of the female broiler chickens.[1]

	Dietary treatments[2]					P[3]	
Variable	**C**	**TM5**	**TM10**	**TM15**	**SEM**	**Linear**	**Quadratic**
Live weight (LW) (g)	1980	2149	2118	2018	33.68	0.771	0.054
Carcass weight (g)	1377	1501	1500	1276	38.80	0.348	0.025
Carcass weight (% LW)	69.46	69.75	70.77	62.86	1.78	0.256	0.266
Breast (g)	332	388	359	353	10.24	0.688	0.135
Breast (% LW)	16.64	17.92	16.94	17.43	0.24	0.512	0.406
Thigh (g)	411	433	434	402	6.00	0.646	0.026
Thigh (% LW)	20.80	20.20	20.51	20.00	0.16	0.163	0.883
Spleen (g)	2.62	2.84	2.83	2.80	0.13	0.672	0.677
Spleen (% LW)	0.13	0.13	0.13	0.14	0.01	0.742	0.768
Liver (g)	25.01	27.30	27.03	25.68	0.82	0.825	0.302
Liver (% LW)	1.26	1.26	1.27	1.26	0.02	0.921	0.941
Gizzard (g)	36.75	38.20	36.83	34.40	1.03	0.392	0.379
Gizzard (% LW)	1.83	1.77	1.74	1.69	0.04	0.168	0.914
Abdominal fat (g)	40.5	43.3	33.8	43.5	1.29	0.014	0.057
Abdominal fat (% LW)	0.66	0.86	1.08	0.98	0.23	0.005	0.074

879 [1]Each mean represents 5 pens with 2 chicks/pen (n = 5/treatment).

880 [2]Four dietary treatments: C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level

881 of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*.

882 [3]Statistical significance: $P < 0.05$.

883

884 **Table 4.** Effect of the dietary TM larvae meal inclusion on the haematological and serum parameters of the female

885 broiler chickens.[1]

	Dietary treatments[2]					P[4]	
Variable[3]	**C**	**TM5**	**TM10**	**TM15**	**SEM**	**Linear**	**Quadratic**
Erythrocyte (10^6 cell/µl)	2.27	2.41	2.47	2.39	0.28	0.073	0.040
Leukocyte (10^3 cell/µl)	9.28	9.07	9.31	9.70	0.23	0.521	0.562
H/L ratio	0.83	0.68	0.75	0.77	0.03	0.632	0.095
Albumin (g/dl)	1.66	1.35	1.27	1.32	0.06	0.046	0.134
Total protein (g/dl)	3.31	3.80	3.85	4.10	0.14	0.068	0.663
GGT (UI/l)	26.86	22.13	21.61	25.56	1.05	0.629	0.046
AST (UI/l)	189.48	227.18	217.11	211.06	8.62	0.494	0.229
ALT (UI/l)	20.06	21.33	18.57	15.90	1.16	0.164	0.428
Uric Acid (mg/dl)	3.44	4.03	3.28	3.35	0.21	0.617	0.562
Creatinine (mg/dl)	0.36	0.38	0.36	0.38	0.00	0.410	0.957
Triglycerides (mg/dl)	42.44	44.48	52.80	36.03	3.05	0.686	0.133
Cholesterol (mg/dl)	60.33	71.12	70.87	77.80	3.71	0.138	0.799
Glucose (mg/dl)	222.60	221.10	219.70	227.00	1.62	0.431	0.196
Phosphorus (mg/dl)	3.67	4.19	3.93	5.56	0.33	0.067	0.376
Magnesium (mEq/l)	1.30	1.15	1.18	1.15	0.03	0.159	0.380
Iron (µg/dl)	102.44	81.20	81.26	103.89	11.64	0.985	0.392

886 [1]Each mean represents 5 pens with 2 chicks/pen (n = 5/treatment).

887 [2]Four dietary treatments: C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level

888 of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*.

889 [3]H/L, heterophiles to lymphocytes ratio; GGT, gamma glutamyl transferase; AST, aspartate aminotransferase;

890 ALT, alanine aminotransferase; n, number of birds.

891 [4]Statistical significance: $P < 0.05$.

892

Table 5. Effects of diet, intestinal segment and interaction between diet and intestinal segment on the intestinal morphometric indices of the female broiler chickens.

Index	Fixed effect	d.f.[3]	F	P[4]
	Diet[1]	3	1.210	0.310
Vh (mm)	Intestinal segment[2]	2	68.115	< 0.001
	Diet × Intestinal segment	6	0.922	0.483
	Diet	3	0.891	0.449
Cd (mm)	Intestinal segment	2	7.275	0.001
	Diet × Intestinal segment	6	1.593	0.157
	Diet	3	0.705	0.551
Vh/Cd (mm/mm)	Intestinal segment	2	35.195	< 0.001
	Diet × Intestinal segment	6	0.277	0.947

[1]Four dietary treatments: C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*.

[2]Three intestinal segments: duodenum, jejunum and ileum.

[3]Degrees of freedom.

[4]Statistical significance: $P < 0.05$.

Vh, villus height; Cd, crypt depth; Vh/Cd, villus height to crypt depth ratio.

902 Table 6. Least square means of intestinal morphometric indices in female broiler chickens in
903 relation to diet and intestinal segment.

Index	Fixed effect	Effect levels	Least square mean[1]	SEM
Vh (mm)	Diet[2]	C	1.73	0.06
		TM5	1.67	
		TM10	1.57	
		TM15	1.61	
	Intestinal segment[3]	DU	2.08[a]	0.05
		JE	1.65[b]	
		I	1.20[c]	
Cd (mm)	Diet	C	0.20	0.01
		TM5	0.20	
		TM10	0.20	
		TM15	0.21	
	Intestinal segment	DU	0.21[a]	0.00
		JE	0.21[a]	
		I	0.19[b]	
Vh/Cd (mm/mm)	Diet	C	8.49	0.34
		TM5	8.51	
		TM10	8.00	
		TM15	8.00	
	Intestinal segment	DU	10.06[a]	0.47
		JE	8.13[b]	
		I	6.56[c]	

904 [1]Means with different superscript letters (a, b) within the same column per fixed effect (i.e.
905 diet, intestinal segment) differ significantly ($P < 0.05$).

[2]C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*

[3]DU = duodenum; JE = jejunum; I = ileum.

Figure legends

Figure 1. Histological findings of the female broiler chickens. **A)** TM5 group. A normal spleen. 5× Haematoxylin & Eosin stain. **B)** TM5 group. Spleen with severe and diffuse depletion of the white pulp. A high number of apoptotic cells (arrowheads) are observed. 20× Haematoxylin & Eosin stain. **C)** C group. A normal follicle in the bursa of Fabricius. 10× Haematoxylin & Eosin stain. **D)** C group. Bursa of Fabricius with mild and multifocal follicular depletion (arrow) associated with intrafollicular cyst (*). 10× Haematoxylin & Eosin stain.