1 **Effects of yellow mealworm larvae (*Tenebrio molitor*)**

2 **inclusion in diets for female broiler chickens: implications**

3 **for animal health and gut histology**

4

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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 ($P <$

74   0.01, maximum with TM15). A quadratic effect (P < 0.05,

75   maximum with TM5) was observed for the carcass weight. The

76   abdominal fat weight and percentage showed a linear response

77   to dietary TM meal inclusion (P < 0.05 and P < 0.01, maximum

78   with TM15 and TM10). A quadratic increase (P < 0.05,

79   maximum with TM10) was observed for the erythrocytes,

80   while the albumin and GGT showed a linear and quadratic

81   decrease (P < 0.05, minimum with TM10) in relation to TM

82   utilization. Gut morphology and histopathological findings

83   were not significantly influenced (P > 0.05) by dietary TM

84   meal inclusion. The present study suggests that increasing

85   levels of dietary TM meal inclusion in female broiler chickens

86   diets may improve body weight and feed intake, but can

87   partially worsen feed efficiency. However, positive effects on

88   carcass traits and haematochemical parameters related to TM

89   meal utilization are observed, along with no negative influence

90   on gut morphology and histological findings.

91

95

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,

145 intestinal morphology and histological features of female

146 broiler chickens fed diets including TM meal.

147

148 **Materials and Methods**

149 *Birds and Husbandry*

150 The present trial was performed in collaboration with a local

151 poultry corporation named "O.R.A. Agricola S.r.l." sited in

152 Cherasco (Cuneo, Italy). The experimental protocol was

153 designed according to the guidelines of the current European

154 and Italian laws on the care and use of experimental animals

155 (European Directive 86 609/EEC, put into law in Italy with

156 D.L. 116/92). Furthermore, the experimental protocol was

157 approved by the Ethical Committee of the Department of

158 Veterinary Sciences of the University of Turin (Italy). A

159 poultry house of 14 m wide × 141 m long × 4.7 m high,

160 equipped with waterproof floor and wall, completely covered

161 by tiles and provided with automatic ventilation system was

162 used.

163 A total of 160 female broiler chicks (Ross 708) at one-day of

164 age were randomly allotted to 4 dietary treatments, each

165 consisting of 5 pens as replicates with 8 chicks per pen. Each

166 pen was 1.20 m wide × 1.20 m long and was equipped with a

167 feeder occupying a surface of almost 1800 cm^2, three nipple

168 drinkers and rice hulls as litter. During the first three weeks, the

169 animals were heated by infrared lamps to maintain the suitable

170 temperature according to standard breeding practices (Aviagen,

171 2014). Lighting schedule was 23 hours light : 1 hour darkness

172 until day 3 and 18 h light : 6 hours darkness until slaughter age.

173 At hatching, all chicks received vaccination against Newcastle

174 disease, Gumboro disease, infectious bronchitis and

175 coccidiosis. Vaccine recalls were performed on day 9 for

176 infectious bronchitis and on day 18 for Gumboro and

177 Newcastle diseases.

178

179 *Diets*

180 A basal diet based on corn meal, corn gluten meal and soybean

181 meal was formulated and served as control (C) group, while 50,

182 100 and 150 g/kg full-fat TM larvae meal (Gaobeidian

183 Shannong Biology CO., LTD, Gaobeidian, Hebei province,

184 China) inclusion as a partial replacement of soybean meal, corn

185 gluten meal and soybean oil constituted the three experimental

186 treatment groups (TM5, TM10 and TM15) (Table 1). TM meal

187 nutritive composition and energy content were the following:

188 dry matter, 939.0 (g/kg as fed); organic matter, 912.0 (g/kg as

189 fed); crude protein (CP), 519.0 (g/kg as fed); ether extract (EE),

190 236.0 (g/kg as fed); 117.0 (g/kg as fed); neutral detergent fibre

191 (NDF); 79.5 (g/kg as fed); acid detergent fibre (ADF); DL-

192 methionine, 10.1 (g/kg as fed); L-lysine, 35.9 (g/kg as fed);

193 gross energy, 24.4; apparent metabolizable energy (AMEn),

194 16.02 (MJ/kg DM). Three different diets were used per each

195 dietary treatments during the three phases of growth: a starter

196 diet (days 1 to 12), a grower diet (days 12 to 25) and a finisher

197 diet (day 25 to 40). For each phase, the experimental diets were

198 isonitrogenous and isoenergetic and were formulated using the

199 AMEn values for TM measured *in vivo* for broiler chickens (De

200 Marco et al., 2015). Diets met or exceeded NRC (1994)

201 requirements and were adjusted according to Aviagen (2014)

202 broiler nutrition specifications. Feed and water were provided

203 *ad libitum*.

204

205 ***Chemical analysis***

206 The diets were ground to pass through a 0.5-mm sieve and

207 stored in airtight plastic containers for DM (method number

208 943.01), ash (method number 924.05), CP (method number

209 954.01), EE (method number 920.39), NDF (method number

210 2002.04) and ADF (method number 973.18) determination

211 (AOAC, 2004).

212

213 ***Growth Performances***

214 Health status and mortality were daily monitored during the

215 whole experimental period. Live weight (LW) of the animals

216 was recorded at an individual level at the beginning of the trial,

217 at day 12, 25 and 40. Average daily gain (ADG) and average

218 daily feed intake (DFI) were recorded at an individual and a

219 pen level, respectively, at the end of each growth period. Feed

220   conversion ratio (FCR) was determined for each growth period

221   and for the overall experimental period. All measurements were

222   made on the pen basis using a high precision electronic scale

223   (Sartorius – Signum®).

224

225   *Slaughtering procedure and recordings*

226   At day 40, all birds were individually weighed and 10

227   broilers/diet (2 birds/pen) were chosen on the basis of pen

228   average LW and identified with a shank ring.

229   All selected animals were slaughtered in a commercial abattoir.

230   The plucked and eviscerated carcasses were obtained and the

231   head, neck, feet and abdominal fat were removed to obtain

232   carcass-for-grilling. The weights of liver, spleen, gizzard and

233   abdominal fat were immediately recorded. All slaughtered

234   carcasses were stocked in a cooling chamber (0-4 °C) for 24 h.

235   Weights of carcass-for-grilling, breast and thighs were

236   successively recorded. Carcass-for-grilling, breast, thigh and

237   organs weights were also expressed as percentage of LW.

238   Collected feet were examined macroscopically using the

239   Swedish FPD scoring system (Ekstrand et al., 1997). According

240   to this system, 0 = no lesion, slight discoloration of the skin or

241   healed lesion; 1= mild lesion, superficial discoloration of the

242   skin and hyperkeratosis; 2 = severe lesion, affected epidermis,

243   blood scabs, hemorrhage and severe swelling of the skin.

244

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 $700 \times g$ 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,

270 glucose, phosphorus, magnesium, iron, uric acid and creatinine

271 serum concentrations were measured by means of enzymatic

272 methods in a clinical chemistry analyzer (Screen Master Touch,

273 Hospitex diagnostics Srl., Firenze, Italy).

274

275 ***Histomorphological investigations***

276 The slaughtered animals were submitted to

277 anatomopathological investigations. Intestinal segment samples

278 (approximately 5 cm in length) of duodenum, jejunum, ileum

279 and caecum were excised from each bird and flushed with 0.9%

280 saline to remove all the content. The collected segments of

281 intestine were the loop of the duodenum, the tract before

282 Meckel's diverticulum (jejunum), the tract before the ileocolic

283 junction (ileum) and the apex of the caeca (caecum). Gut

284 segments were fixed in Carnoy's solutions for morphometric

285 analysis. Tissues were routinely embedded in paraffin wax

286 blocks, sectioned at 5 μm thickness, mounted on glass slides

287 and stained with Haematoxylin & Eosin (HE). The evaluated

288 morphometric indices were Vh (from the tip of the villus to the

289 crypt), Cd (from the base of the villus to the submucosa) and

290 the Vh/Cd ratio (Laudadio et al., 2012). Morphometric analyses

291 were performed on 10 well-oriented and intact villi and 10

292 crypts chosen from duodenum, jejunum and ileum (Qaisrani et

293 al., 2014). Samples of liver, spleen, thymus, bursa of Fabricius,

294 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

320　model (GLM). The GLM allowed the morphometric indices

321　(Vh, Cd and Vh/Cd, separately) to depend on three fixed

322　factors (diet, intestinal segment and interaction between diet

323　and intestinal segment). The interactions between the levels of

324　the fixed factors were evaluated by pairwise comparisons.

325　Statistical analysis was performed by procedure "General

326　Linear Models > Univariate". Histopathological and FPD

327　scores were analyzed by Kruskal-Wallis test (post-hoc test:

328　Dunn's Multiple Comparison test). P values < 0.05 were

329　considered statistically significant. The results were expressed

330　as mean and pooled standard error of the mean (SEM).

331

332　**Results**

333　*Growth performance*

334　No clinical signs were observed and the birds remained healthy

335　during the whole experimental period. The mortality rates of C

336　(2.5%), TM5 (2.5%), TM10 (0%) and TM15 (2.5%) groups

337　were not significantly different among treatments (P > 0.05).

338　Growth performance of the broiler chickens are summarized in

339　Table 2. At 12 days of age, the LW increased linearly with

340　increasing TM meal levels (P < 0.05) and the linear response

341　increased to a maximum corresponding to the inclusion of 150

342　g/kg of TM meal. At 25 days of age, no significant effects

343　related to TM meal utilization were observed. At 40 days of

344　age, the LW showed quadratic response to increasing TM meal

14

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***

15

370   Table 3 summarizes the slaughtering performance of the broiler

371   chickens. The carcass weight increased quadratically with

372   increasing TM meal levels (P < 0.05) and the quadratic

373   response increased to a maximum corresponding to the

374   inclusion of 50 g/kg of TM meal. The abdominal fat weight

375   showed linear response to increasing TM meal levels (P <

376   0.05), with a maximum corresponding to the inclusion of 150

377   g/kg of TM meal. Similarly, the abdominal fat percentage

378   increased linearly with increasing TM meal levels (P < 0.01)

379   and the linear response increased to a maximum corresponding

380   to the inclusion of 100 g/kg of TM meal. On the contrary, no

381   significant effects related to TM meal utilization were observed

382   for the other carcass traits (P > 0.05). FPD scores (C: 0.40;

383   TM5: 0.20; TM10: 0.20; TM15: 0.00) were also not influenced

384   by dietary TM meal inclusion (P > 0.05).

385

386   ***Haematological and serum parameters***

387   Haematological and serum biochemical traits of the broiler

388   chickens are summarized in Table 4. The erythrocytes

389   increased quadratically with increasing TM meal levels (P <

390   0.05) and the quadratic response increased to a maximum

391   corresponding to the inclusion of 100 g/kg of TM meal. The

392   albumin showed linear response to increasing TM meal levels

393   (P < 0.05), with a minimum corresponding to the inclusion of

394   100 g/kg of TM meal. Similarly, the GGT decreased

395   quadratically with increasing TM meal levels ($P < 0.05$) and the

396   quadratic response decreased to a minimum corresponding to

397   the inclusion of 100 g/kg of TM meal. On the contrary, no

398   significant effects related to TM meal utilization were observed

399   for the other blood and serum parameters ($P > 0.05$).

400

401   *Histomorphological investigations*

402   The effects of the diet, intestinal segment and interaction

403   between diet and intestinal segment on gut morphometric

404   indices of the broiler chickens are shown in Tables 5 and 6.

405   There was no influence of diet or interaction between diet and

406   intestinal segment ($P > 0.05$) on the gut morphometric indices.

407   On the contrary, Vh, Cd and Vh/Cd depended on intestinal

408   segment ($P < 0.001$, $P = 0.001$ and $P < 0.001$, respectively)

409   (Table 5). In particular, the duodenum showed higher Vh ($P <$

410   0.001) than the jejunum and ileum. Furthermore, higher Cd (P

411   < 0.001) was found in the duodenum and jejunum than the

412   ileum. Similarly, the duodenum and jejunum showed higher

413   Vh/Cd ($P < 0.001$) than the ileum (Table 6).

414   Histopathological alterations were observed in all the dietary

415   treatments and developed in spleen, thymus, bursa of Fabricius

416   and liver, while heart and kidney showed no significant

417   findings. Spleen (C = 1.50 ± 0.22; TM5 = 1.20 ± 0.25; TM10 =

418   1.10 ± 0.31; TM15 = 1.10 ± 0.28), thymus (C = 0.10 ± 0.10;

419   TM5 = 0.30 ± 0.15; TM10 and TM15 = 0.10 ± 0.10), bursa of

420   Fabricius (C = 1.90 ± 0.10; TM5 = 1.50 ± 0.27; TM10 = 1.80 ±

421   0.20; TM15 = 1.70 ± 0.21) and liver (C = 0.90 ± 0.23; TM5 =

422   1.10 ± 0.23; TM10 = 0.90 ± 0.23; TM15 = 0.30 ± 0.15) scores

423   were not influenced by dietary TM meal inclusion. Spleen

424   showed moderate (C = 30% of the broilers; TM5 = 40%; TM10

425   = 10%; TM15 = 30%) to severe (C = 60%; TM5 = 40%; TM10

426   = 50%; TM15 = 40%) white pulp depletion or hyperplasia. The

427   10% I, 20% (TM5), 40% (TM10) and 30% (TM15) of the

428   animals had normal spleen (Fig. 1A-B). In thymus, moderate

429   cortical depletion was found in all the groups (C = 10% of the

430   broilers; TM5 = 30%; TM10 and TM15 = 10%). A normal

431   thymus was observed in 90% I, 70% (TM5) and 90% (TM10

432   and TM15) of the animals. Bursa of Fabricius showed moderate

433   (C and TM5 = 10% of the broilers; TM10 = 0%; TM15 = 10%)

434   to severe (C = 80%; TM5 = 70%; TM10 = 90%; TM15 = 80%)

435   follicular depletion. The 0% I, 20% (TM5), 10% (TM10) and

436   10% (TM15) of the animals had normal bursa of Fabricius (Fig.

437   1C-D). In liver, moderate (C, TM5 and TM10 = 50% of the

438   broilers; TM15 = 30%) to severe (C = 20%; TM5 = 30%;

439   TM10 = 20%; TM15 = 0%) perivascular lymphoid tissue

440   activation. A normal liver was observed in 30% I, 20% (TM5),

441   30% (TM10) and 70% (TM15) of the animals.

442

443   **Discussion**

444   *Growth performances*

445 Growth performances of the broiler chickens of the present

446 study were consistent with the reference values recorded in the

447 commercial farm in which the trial was conducted.

448 The body weight, weight gain and feed intake of the birds in

449 the present trial improved with increasing levels of TM meal

450 inclusion, but the feed efficiency resulted partially impaired.

451 Little information on the influence of dietary TM meal

452 inclusion in broiler chickens is currently available. Ramos-

453 Eldoury et al. (2002) and Biasato et al. (2016) did not show any

454 effects for the growth performance in fast-growing and

455 intermediate-growing chickens, respectively, fed diets in which

456 the TM inclusion level ranged from 50 to 100 g/kg. Differently,

457 Ballitoc and Sun (2013) and Bovera et al. (2015) observed

458 improved growth performance in fast-growing chickens fed

459 diets with low (from 5 to 100 g/kg) or high (296 g/kg) TM

460 inclusion levels, respectively. Similar findings were obtained

461 by Loponte et al. (2017) in partridges fed diets in which the TM

462 inclusion level ranged from 250 to 500 g/kg. Recently, Islam

463 and Yang (2017) also found a positive effect of a mealworm-

464 based probiotic on broiler growth performance. Some authors

465 also explored the possibility to use other insect meals in poultry

466 feeding. Adeniji (2007) and Hwangbo et al. (2009) studied the

467 effects of housefly-maggots as feed supplement in the diet of

468 broiler chickens: the first found no differences for growth

469 performance with inclusion levels ranging from 55 to 220 g/kg,

19

470 while the latter (inclusion rate: 50-200 g/kg) observed a linear

471 increase in LW gain. Ijaiya and Eko (2009) evaluated the

472 effects of replacing dietary fishmeal with silkworm meal

473 (inclusion rate: 22-93 g/kg) on growth performance of broiler

474 chickens, finding no differences related to insect meal

475 utilization. Also Oyegoke et al. (2006) and Wang et al. (2005)

476 observed no adverse effects on growth performance of broiler

477 chickens fed diets with *Cirina forda* (inclusion rate: 20-40

478 g/kg) and *Gryllus testaceus* (inclusion rate: 50-150 k/kg),

479 respectively. Cullere et al. (2016) recently studied the influence

480 of the inclusion (from 100 to 150 g/kg) of *Hermetia illucens*

481 meal in quail diet, finding no differences for growth

482 performances. Schiavone et al. (2017b) also evaluated the

483 effects of replacing soybean oil with *Hermetia illucens* meal

484 (inclusion rate: 500-1000 g/kg) on growth performance of

485 broiler chickens, finding no differences related to insect meal

486 utilization. The wide variability of the results obtained in the

487 previous studies may be related to the nutritive value of the

488 insect meal used, which can be influenced by the species, the

489 insect life stage (adult, larva or pupa) and the insect rearing

490 substrate (Sànchez-Muros et al., 2014).

491 The improvement of feed intake observed in the birds fed TM

492 diets of the present trial was considered suggestive of increased

493 feed palatability in relation to the addition of yellow

494 mealworms, since insects are naturally consumed by wild birds

495 and free-range poultry (Zuidhof et al., 2003). In particular, the

496 increased DFI observed in the starter period, which was

497 accompanied by increased LW and ADG and unaffected FCR,

498 was quite relevant. Indeed, starter period (from hatch to 10

499 days) is considered the most important in broiler production,

500 since growth and development take place at an incredible rate

501 during it. In this period, the chicks' weight quadruples, thus

502 influencing the following growth rate (Aviagen, 2014). On the

503 contrary, the increased DFI observed in the growing period was

504 accompanied by unaffected LW and ADG and subsequently

505 impaired FCR, thus representing a negative effect related to

506 TM meal utilization. De Marco et al. (2015) speculated that the

507 chitin contained in the exoskeleton of the TM meal may

508 negatively influence the apparent digestibility coefficient of the

509 total tract of nutrients. Furthermore, Ravindran and Blair

510 (1993) pointed out that the chitin of insects is difficult to digest

511 by domestic poultry. As suggested by Rumpbold and Schlüter

512 (2013), the partial chitin removal through high pressure

513 processing could improve the use of insects as feeding

514 ingredient thanks to disruption of the link between some chitin-

515 bound proteins. However, the limited number of birds included

516 in the current trial could have influenced the data interpretation.

517 The results obtained need to be confirmed on a larger number

518 of animals.

519

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

569 accompanied by the increase of albumin/globulin ratio, which

570 was also reported by Bovera et al. (2015) and ascribed to the

571 properties of chitin contained in insect meal. High globulin

572 concentrations and low albumin/globulin ratios generally

573 indicate better disease resistance and immune response of birds

574 (Griminger and Scanes, 1986). Another interesting finding is

575 represented by the decrease of GGT serum levels in TM

576 animals. Indeed, a high GGT concentration in birds is used as

577 an indicator of liver disease and bile flow disorders (Ognik and

578 Krauze, 2016). Therefore, GGT reduction can be considered a

579 positive effect related to TM meal utilization.

580

581 *Histomorphological investigations*

582 Dietary TM meal inclusion did not influence the gut

583 morphology of the birds of the present study, as already

584 observed by Biasato et al. (2016). Morphometric measurements

585 of Vh and Cd are generally used to assess intestinal

586 development (Franco et al., 2006), since represent useful

587 indicators of gut proliferative and absorptive compartments

588 (Lenhardt and Mozes, 2003). The Vh/Cd ratio is also evaluated,

589 because it gives an indication of the likely maturity and

590 functional capacity of the enterocytes (Hampson, 1986). As

591 previously reported (Uni et al., 1999; Iji et al., 2001; Biasato et

592 al., 2016), the present study confirms that both duodenum and

593 jejunum show a greater morphological development compared

594   with the ileum. Indeed, the duodenum is the intestinal tract with

595   the fastest cell renewal, and is also the first segment of the

596   small intestine to receive physical, chemical and hormonal

597   stimuli provoked by diet (Macari, 1998). Furthermore, the

598   jejunum is an important site for nutrient digestion (Iji et al.,

599   2001). Therefore, dietary TM inclusion preserves a

600   proximodistal decreasing gradient of the morphometric indexes

601   from the duodenum to the ileum, thus suggesting the

602   maintenance of the physiological gut development.

603   The broiler chickens of the present trial showed different

604   degrees of lymphoid system activation, with no differences

605   related to dietary TM meal inclusion. This result could be

606   related to the stress occurrence in modern poultry rearing

607   operations. Stress can be caused by a variety factors,

608   physiological (rapid growth rate) and social (overcrowding)

609   ones included (Liles et al., 2015). However, a great deal of

610   individual bird variability in some immunological measures

611   (i.e., stimulation index, heterophils to lymphocyte ratio and

612   lymphocyte blastogenesis) may also be considered (Talebi et

613   al., 1995).

614

615   **Conclusion**

616   In conclusion, the present study suggests that increasing levels

617   of dietary TM meal inclusion in female broiler chickens diets

618   may improve body weight, weight gain and feed intake, but can

25

619   partially worsen feed efficiency. However, positive effects on

620   carcass traits and haematochemical parameters are observed,

621   along with no negative influence on gut morphology and

622   histological findings. These results confirm previous data

623   concerning the safety of TM utilization in poultry feed, even if

624   legislative issues are still needed to allow insect meal to be

625   used as transformed animal protein to feed monogastric farm

626   animals.

627

637

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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

[1]Mineral-vitamin premix (Final B Prisma, IZA SRL), given values are supplied per kg of diet: 2.500.000 IU of vitamin A; 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 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 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 mg of Se.

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

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

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

Variable[2]	Age	Dietary treatments[1]					P[3]	
		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]

| Variable | Dietary treatments[2] | | | | | P[3] | |
	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*.

41

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]

| Variable[3] | Dietary treatments[2] | | | | | P[4] | |
	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).

43

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

893

894   **Table 5.** Effects of diet, intestinal segment and interaction between diet and intestinal segment on the intestinal

895   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

896   [1]Four dietary treatments: C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level

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

898   [2]Three intestinal segments: duodenum, jejunum and ileum.

899   [3]Degrees of freedom.

900   [4]Statistical significance: P < 0.05.

901   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	
		TM5	1.67	
		TM10	1.57	0.06
		TM15	1.61	
	Intestinal segment[3]	DU	2.08^a	
		JE	1.65^b	0.05
		I	1.20^c	
Cd (mm)	Diet	C	0.20	
		TM5	0.20	
		TM10	0.20	0.01
		TM15	0.21	
	Intestinal segment	DU	0.21^a	
		JE	0.21^a	0.00
		I	0.19^b	
Vh/Cd (mm/mm)	Diet	C	8.49	
		TM5	8.51	
		TM10	8.00	0.34
		TM15	8.00	
	Intestinal segment	DU	10.06^a	
		JE	8.13^b	0.47
		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 ($P < 0.05$).

906   ^{2}C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of

907   *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*

908   ^{3}DU = duodenum; JE = jejunum; I = ileum.

909 **Figure legends**

910

911 **Figure 1.** Histological findings of the female broiler chickens. **A)** TM5 group. A normal

912 spleen. 5× Haematoxylin & Eosin stain. **B)** TM5 group. Spleen with severe and diffuse

913 depletion of the white pulp. A high number of apoptotic cells (arrowheads) are observed. 20×

914 Haematoxylin & Eosin stain. **C)** C group. A normal follicle in the bursa of Fabricius. 10×

915 Haematoxylin & Eosin stain. **D)** C group. Bursa of Fabricius with mild and multifocal

916 follicular depletion (arrow) associated with intrafollicular cyst (*). 10× Haematoxylin &

917 Eosin stain.