Evaluation of an insect meal of the Black Soldier Fly (Hermetia illucens) as soybean substitute: Intestinal morphometry, enzymatic and microbial activity in laying hens

Evaluation of an insect meal of the Black Soldier Fly (Hermetia illucens) as soybean substitute: Intestinal morphometry, enzymatic and microbial activity in laying hens

Monica I. Cutrignelli, Maria Messina, Francesca Tulli, Basilio Randazzo, Ike Olivotto, Laura Gasco, Rosa Loponte, Fulvia Bovera

PII: S0034-5288(17)30638-0
DOI: doi:10.1016/j.rvsc.2017.12.020
Reference: YRVSC 3492

To appear in: *Research in Veterinary Science*

Received date: 9 June 2017
Revised date: 20 December 2017
Accepted date: 26 December 2017

Please cite this article as: Monica I. Cutrignelli, Maria Messina, Francesca Tulli, Basilio Randazzo, Ike Olivotto, Laura Gasco, Rosa Loponte, Fulvia Bovera , Evaluation of an insect meal of the Black Soldier Fly (Hermetia illucens) as soybean substitute: Intestinal morphometry, enzymatic and microbial activity in laying hens. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Yrvsc(2017), doi:10.1016/j.rvsc.2017.12.020

Evaluation of an insect meal of the Black Soldier Fly (Hermetia illucens) as soybean substitute: intestinal morphometry, enzymatic and microbial activity in laying hens

Monica I. Cutrignelli[a], Maria Messina[b], Francesca Tulli[b], Basilio Randazzo[c], Ike Olivotto[c], Laura Gasco[d], Rosa Loponte[a], Fulvia Bovera[a]

[a]University of Napoli Federico II, Department of Veterinary Medicine and Animal Production, via F. Delpino, 1, 80137 Napoli, Italy

[b]University of Udine, Departmentof AgriFood, environment and Animal Science, via Sondrio, 2 33100 Udine, Italy

[c]University Politecnica delle Marche, Department of Life and Environmental sciences, via Brecce Bianche, 60131 Ancona, Italy

[d]Department of Agricultural, Forest, and Food Sciences, University of Torino, largo Braccini 2, 10095 Grugliasco, Italy

Corresponding author: Prof. Fulvia Bovera, bovera@unina.it

Abstract.

This research investigated the ileum morphometry and enzymatic activity, the caecal volatile fatty acid production and the apparent nutrient digestibility in laying hens fed a *Hermetia illucens* larvae meal (HILM) as a complete replacement of diet soybean meal (SBM). The hens fed HILM exhibited a lower live weight ($P<0.05$) and a higher incidence of the full digestive tract ($P<0.05$) than the SBM group. In the duodenum, the maltase exhibited a higher ($P<0.05$) activity in the HILM group while the intestinal alkaline phosphatase (IAP) had a higher ($P<0.05$) activity in the SBM group. In the ileum, the maltase and saccarase had a higher activity in the HILM hens ($P\leq0.01$) while the IAP and ɤ glutamil transferase had a higher activity in the SBM group ($P<0.05$ and $P<0.01$, respectively). The HILM group showed a higher ($P<0.05$) villi height in the duodenum, while the opposite happened in the jejunum and the ileum. Only in the ileum the crypt depth resulted higher ($P<0.05$) in the HIML group than in the SBM. The higher production of acetate ($P<0.05$) and butyrate ($P<0.01$) affected the total production of volatile fatty acids of the HILM group. The coefficient of apparent digestibility of dry and organic matter as well as of crude protein were higher ($P<0.05$) in SBM group. The total replacement of SBM with HILM in laying hens diet from 24 to 45 weeks of age resulted in a higher caecal production of butyric acid while the enzymatic activities of brush border membrane were partially reduced.

Key words: poultry, *Hermetia illucens*, nutrient digestibility, gut morphometry, brush border membrane, volatile fatty acid

Introduction

Due to their nutritive value and environmental-friendly production (Oonincx et al., 2010) there is an increasing interest on insects as potential food and feed source (Makkar et al., 2014; Pal and Roy, 2014). The potential use of insects as an alternative protein source is particularly applicable in poultry as their "natural" diet normally includes insects (Bovera et al., 2015). On this regard, several studies are available on the effect of meals obtained from larvae of different insect species, mainly *Tenebrio molitor* and *Hermetia illucens* (Oyegoke et al., 2006; De Marco et al., 2015; Biasato et al., 2016; Bovera et al., 2016; Loponte et al., 2017) on bird growth performance, blood profiles and meat quality. On the contrary, very few studies are available on laying hens, in particular raised in cage systems, due to the length of the production cycle and the objective problem to manage a completely automated system. Wang et al. (1996) observed that dried ground mealworms as replacement of fishmeal in laying hens diet increased the egg production by 2.4 %. Agunbiade et al. (2007), in a study on 50 weeks old laying hens, showed that the maggot meal could replace 50% of fishmeal protein (5% in diet) without adverse effects on the egg production and shell strength but 100% of replacement decreased the egg production. More recently, Maurer et al. (2016) showed that the *Hermetia illucens* larvae meal included up to 100% on protein basis in a laying hens diet did not affect the egg production, feed intake and feed conversion efficiency.

The dietary full replacement of the soybean meal with a defatted insect meal from *H. illucens* larvae significantly decreased the feed intake and the productive performance of the laying hens from 24 to 45 weeks of age (Marono et al., 2017). The authors attributed this negative effect to the color (darker) and the flavor of the insect meal.

At the present, the insect meals obtained from the different insects have an excessive cost and are not competitive compared to the other protein sources as the soybean meal. This is due to a non-consistent industrial production of the insect meals, in particular in Europe as the Regulation EC 999/2001 banned the use of the processed animal protein (PAP) in animal nutrition. However, the European law is evolving. Regulation 56/2013 (approved since the first June 2013) providing that

aquaculture animals can be fed with animal proteins derived from non-ruminants, and recently the Regulation EU 2017/893 approved the use of PAP derived from seven insect species in aquaculture. *H. illucens* is among the authorized species. As far as poultries are concerned, stakeholders are confident and the authorization of the use of the insect PAP is expected within 2020 – 2022.

The present research is the pursuance of the trial by Marono et al. (2017) and aims to verify the potential effects of the insect meal from *Hermetia illucens* larvae on the nutrient digestibility, intestinal morphometry, histology, enzymatic and bacterial activities of 45 weeks old laying hens under intensive cage production system.

Material and methods

All the animals were humanely treated according to the principles stated by the EC Directive 63/2010/EEC regarding the protection of the animals used for experimental and other scientific purposes. The experimental procedures received the approval from the Ethical Animal Care and Use Committee of the Department of Veterinary Medicine and Animal Production of the University of Napoli Federico II, Italy (prot. N. 2017/0017676).

The trial was carried out in a private farm located in southern Italy, along 21 weeks. A total of 108, 24 weeks old Lohman Brown Classic laying hens (average live weight 1.78 ± 0.15 kg) were randomly allotted into 2 groups (54 hens/group). The hens were housed in the same building in modified cages (800 cm^2/hen), under controlled temperature and humidity conditions. For each group, hens were distributed in 3 cages (18 hens/cage) and each cage was divided by two internal transects in three equal areas, to obtain 9 replicates of 6 hens per group. Feed and water were manually distributed, and appropriate separations were placed along the trough and the line of egg collection to control the feed intake and the egg production per each replicate. The dark:light cycle was 9:15 hours.

The two groups were fed two isoprotein and isoenergetic diets, differing in the ingredients used as

main protein source. The soybean meal (SBM) group was fed a corn-soybean meal based diet, while the second group was fed a diet in which the soybean was completely replaced by a defatted meal from *Hermetia illucens* larvae (Hermetia Deutschland GmbH & Co KG, Baruth/Mark, Germany) (HILM group). The diets were formulated to meet hens requirements according to Lohmann Brown classic Management Guide (2011). Celite® (Sigma-Aldrich, St. Louis, Mo) was added at 20 g/kg to the diets and used as an indigestible marker to estimate the nutrient digestibility coefficients. The chemical characteristics of the insect and soybean meals (Table 1) and those of the diets (Tables 2) were determined according to AOAC (2004). The metabolizable energy of the diets was calculated according to the NRC (1994) procedure of estimation. Data on aminoacids, minerals and metabolizable energy of all the ingredients were supplied by the respective producers and used to calculate the correspondent contents in the diets. The amount of protein linked to acid detergent fibre (ADF) was determined (AOAC, 2004) and, only for the insect meal, it was used to estimate the amount of chitin, according to Marono et al. (2015): chitin (%) = ash free ADF (%) – ADF-linked protein (%).

The mash diets and fresh water were administered *ad libitum*. The feed intake was measured over 21 weeks.

At 45 weeks of age, two hens randomly chosen per replicate (18 per group) were slaughtered, the digestive tract was excised, weighed and the different intestinal tracts were identified, and their length was measured. The ileum was separated from 20 mm after Meckel's diverticulum to 40 mm proximal to the ileocecal junction to avoid the contamination of the other intestinal contents and the digesta were pooled per replicate (1 pool from 2 hens per replicate; 9 pools per group), immediately frozen, and subsequently freeze-dried. The dried ileal digesta were ground to pass a 1-mm sieve and stored at –20°C until the chemical analysis. The apparent ileal digestibility coefficients of dry matter (DM), organic matter (OM), crude protein (CP) and ether extract (EE) were measured by the acid insoluble ash (AIA) method (Vogtmann et al., 1995) using the celite as internal marker and estimated using the following equation: 100 – 100 × [(% AIA in the diet/% AIA in the ileal content)

× (% nutrient in the ileal content/% nutrient in the diet)].

The small intestine tracts were washed with an ice-cold isotonic saline buffer (pH7) blotted with absorbent paper and divided into three segments, duodenum, jejunum and ileum. Each tract was weighed and separately wrapped in aluminum foil and stored at -20°C until the analysis of the activity of the brush border membrane (BBM) enzymes.

Villus and Crypt Morphometry

The intestinal samples (0.5 cm) from duodenum, jejunum and ileum were fixed by immersion in 4% phosphate-buffered paraformaldehyde for 48 h. The samples were then washed in a phosphate-buffered saline solution, dehydrated in an ethanol series and embedded in paraffin. Cross sections were stained with Mayer's hematoxylin and eosin and examined under photomicroscope (Olympus Vanox photomicroscope, Japan) for the histopathological assay. For the analysis of the villus height and crypts depth ten microscopic fields of the duodenum, jejunum and ileum were measured for each bird using a calibrated ocular micrometer.

Brush Border Membrane enzymes activity

The BBM enzymes were obtained according to Shirazy-Beechey et al. (1991) with some modifications. All the steps in the procedure were performed at 4°C. Briefly, 100 mg of the tissue, were diluted 1:10 with a buffer (100 mM mannitol, 2 mM Hepes-tris, pH 7.1), added with $MgCl_2$ at a final concentration of 10 mM, and crushed with a tissue-lyser (Tissue Lyser II, Qiagen, Germany) at 30 Hz for 1 minute. The samples were centrifuged at 2,000 x g at 4°C for 10 min and the supernatant was transferred in a new vial and centrifuged at 15,000 x g at 4°C for 10 min. The resulting supernatant was maintained at -20°C until the analysis of the BBM enzyme activity.

The hydrolysis of sucrose and maltose by the mucosal maltase and sucrase, was determined according to Tibaldi et al. (2006).

The intestine alkaline phosphatase (IAP) and γ-glutamyl transpeptidase (γ-GT) activity was determined on the supernatant using two commercial kits (Paramedical, Pontecagnano Faiano, Sa, Italy) as indicated by the manufacturer.

The total protein concentration was determined according to Bradford et al. (1976) (Sigma-Aldrich cat. no. B6916) and bovine serum albumin (Sigma-Aldrich cat. no. 0834) as a standard.

One unit (U) of enzyme activity is the amount of enzyme that transforms or hydrolyses 1 μmole of the substrate per minute. The specific enzyme activity was calculated as 1 U of the enzyme activity per mg of protein.

Volatile fatty acids

The caeca were tied at both ends, separated by sterile instruments from the rest of the gastrointestinal tract, placed in tightly closed plastic bags and put in pre-warmed thermos. After the sampling, the material was transported as soon as possible (about 1 h) to the laboratory, where two quotes of the caecal content (each about 5 ml) were used for the volatile fatty acids (VFAs) determination. The samples were diluted with oxalic acid (1:1, v/v) and VFAs were analyzed by a gas chromatography method (Stanco et al., 2003) (Thermo-Electron mod. 8000top, FUSED SILICA Gaschromatograph, ThermoElectron Corporation, Rodano, Milan, Italy) equipped with OMEGAWAX 250 fused silica capillary column 30 m X 0.25 mm X 0.25 mm film thickness, flame ionisation detector (185 °C), carrier helium (1.7 ml/min) under isothermal condition (125 °C).

Statistical Analysis

The data were processed by ANOVA using the PROC GLM (SAS, 2000). The differences between the groups were analyzed by one-way ANOVA according to the following model: Yij = m + Di + eij, where Y is the single observation, m the general mean, D the effect of the diet (i = HILM or SBM), e is the error. The comparison between the means was performed by Tukey's test (SAS,

2000).

Results

The amount of ADF-linked protein in the insect larvae meal was 5.59% as fed. Therefore, the estimated chitin content resulted 5.40% as fed (46.22% of ADF).

As reported by Marono et al. (2017), the feed intake of hens along the experimental period was higher in the SBM than in HILM group (125.1 vs. 108.0 g/d, $P < 0.001$)

The live weight of the hens at the slaughter age and the intestinal weights and lengths expressed as percentage of the live weight are shown in Table 3. The hens fed *H. illucens* exhibited a lower live weight ($P < 0.05$) and a higher incidence of the full digestive tract ($P < 0.05$) than the ones fed soybean meal.

The table 4 shows the specific activity of maltase, saccarase, IAP and ɤ-GT measured in the different digestive tracts of the hens. In the duodenum, the maltase had a higher ($P < 0.05$) activity in the HILM than the SBM group while the opposite happened for the alkaline phosphatase ($P < 0.05$). In the ileum there are differences between the groups for all the tested enzyme activities. In particular: maltase and saccarase had a higher activity in the hens fed *Hermetia illucens* ($P \leq 0.01$) while IAP and ɤ-GT had a higher activity in the SBM group ($P < 0.05$ and $P < 0.01$, respectively).

The intestinal morphometry was measured in the three tracts of the small intestine of the hens (Table 5). In the duodenum the HILM group showed a higher ($P < 0.05$) villi height than the SBM group, while the opposite happened in the jejunum. In the ileum, the villi height was higher in the SBM group ($P < 0.05$) and the opposite was for the crypt depth. Consequently, the villi to crypt ratio was higher ($P < 0.05$) in the SBM than in the HILM group.

The volatile fatty acid production in the caeca (Table 6) indicated a higher ($P < 0.01$) total VFA production in the HILM group, mainly due to a higher production of acetate ($P < 0.05$) and butyrate ($P < 0.01$), however, no differences were found between groups when they were expressed as percentage of the total VFA.

The coefficients of the apparent ileal digestibility (Table 7) showed a higher digestibility of the dry and organic matter ($P < 0.05$) in the SBM group and the principal contribution to this result was for the crude protein digestibility. The ether extract digestibility was unaffected by the dietary treatments.

Discussion

The full replacement of the dietary soybean meal by a larvae meal from *Hermetia illucens* is responsible of several modifications in the intestinal tracts of laying hens. The reduced coefficients of the apparent ileal digestibility of dry and organic matter in the layers fed the HILM diet (- 4.3 and -4.2 % than SBM group, respectively) were mainly due to the strong reduction of the protein digestibility (-13.6 %). This is related to the presence of the chitin in the insect meal that, as well known, negatively affects the protein digestibility (Longvah et al., 2011). Even if not significant, also the ether extract digestibility was decreased by 5.6 % in comparison to the SBM group. The reduction on the nutrient digestibility associated to the lower feed intake observed in hens fed insects, are responsible of the lowest body weight of hens at the end of the trial. The different colour and flavour of the feed could affect the hens feed intake as observed by Esmail (2013) and Marono et al. (2017).

In the present trial, the dietary treatment did not affect the macroscopic characteristics of the different intestinal tracts as the weight and the length of the duodenum, jejunum and ileum were not different between the groups. Based on the current knowledge, very few studies are available in the literature on the effect of the insect meals on the intestinal traits and are mainly focused on growing broiler. Ballitoc and Sungo (2013) found that the small intestine weight was increased when broilers were fed ground yellow mealworms up to a 10% inclusion level in comparison to the control, but no differences were observed among the other intestinal tracts. In a previous study with broilers fed *Tenebrio molitor* larvae meal as total replacement of the soybean meal, Bovera et al. (2016) found a greater full intestinal length in the birds fed the insects, mainly due to a greater length of the ileum

and ceca. The lack of effects of the dietary treatment on the intestine traits observed in the present trial, can be attributed to the animals age. As known, the development of the digestive system in poultry (including villi size and area) is very rapid in the days 1 and 2 post-hatch and reach a plateau at 5 – 10 d (Uni et al., 1995 and 1996). After this period, the growth of the gastro-intestinal tract is isometric until the somatic maturity (Uni et al., 1998). In our trial, intestinal traits were measured at 45 weeks of age when both the development of the gastrointestinal tract and the animal growth were completed.

The effect of feeding HILM diet on the volatile fatty acid profile of the caeca was evident and indicated an increase of 36.8 % of the total production of VFA in comparison to the control group. In particular, the acetic acid production increased by 36.1 % and the butyrate showed an increase of 62.6 % in the HILM than the SBM group. Similarly to our results, Loponte et al. (2016) found an increased amount of the total VFA (+45.6 %), acetate (+40.3 %) and butyrate (+64.6 %) in broilers fed a *Tenebrio molitor* larvae meal as complete replacement of the soybean meal. The high concentration of the volatile fatty acids indicates that the fermentations by obligate anaerobic bacteria are consistent (Barnes, 1979). This result is particularly interesting considering that the feed intake in hens fed insects was lower than that of the SBM group and thus the amount of substrate available for bacteria fermentation in the intestine of hens from HIML group was lower than the control.

The butyric acid is considered the main enterocytes energy source (Bovera et al., 2010) and is also necessary for a proper development of the Gut-Associated Lymphoid Tissue (Mroz, 2005). It is documented that the butyrate is the major intestinal energy source even when other fuel sources (glucose or glutamine) are available and could stimulate the growth of the colorectal and ileal mucosal cells (Topping and Clifton, 2001; Montagne et al., 2003). This is important for maintaining the function of the entire gastro-intestinal tract, not just of the caeco-colon (Montagne et al., 2003). When a higher amount of butyric acid is available, the increase of the nutrients for enterocytes enhances the blood flow through the intestine and then the tissue oxygenation and the nutrient

transport and absorption (Mahdavi and Torki, 2009). The mechanism of action may involve the local neural networks as well as the chemo-receptors together with direct effects on the smooth muscle cells (Mroz, 2005). In humans, Säemann et al. (2000) demonstrated an anti-inflammatory property of the butyrate, which may have broad implications for the regulation of the immune response; however, the association of the butyrate with the immune and anti-inflammatory response in poultry is limited (Khempaka et al., 2011). Van der Wielen et al. (2000) reported that the volatile fatty acids have a bacteriostatic effect against some enteric bacteria, including *Salmonella typhimurium*, and they did not inhibit the beneficial bacteria of the gastrointestinal tract, such as *Lactobacillus*, in chickens. In addition, the same authors found a negative correlation between the acetate levels and CFU of *Enterobacteriaceae* (including Salmonella spp.) in broiler.

A significant negative correlation between the caecal propionate concentrations and Salmonella colonization in young chickens has been also reported (Nisbet et al., 1996). Moreover, van der Wielen et al. (2000) reported that the increased concentrations of the butyric acid were related to the decreased amounts of Enterobacteriaceae. Therefore, the high concentration of the butyric acid produced in the caeca of hens fed insect meal may play an important part in the mechanism that inhibits *E. coli* and Salmonella colonization.

Unfortunately, sufficient information is not available in the literature on the effects of feeding insect meal on the intestinal microflora and volatile fatty acids production of hens, but our results can be ascribed to the amount of chitin fed by birds of HILM group due to its ability to act as prebiotic (Ballitoc and Sungo, 2013). This agrees with Khempaka et al. (2011) who reported that the inclusion of shrimp head meal as chitin source at 15 and 20 % as feed, as well as the addition of 1.9 % of purified chitin in the broiler diet significantly increased the production of the butyric volatile fatty acid in the caeca.

Standing our estimation, the amount of chitin in the insect meal used in the present trial was 5.40 % as feed corresponding to 46.2 % of ash free acid detergent fiber, in line with the finding of Finke

(2007) who indicated that the ADF fraction in the insects contains an amount of protein from 9.3 to 32.7 % and the amount of chitin ranges from 2.7 to 49.8 g/kg.

However, the highest butyrate amount in the caecal content of the hens fed the HILM diet had contradictory effects on the different small intestine tracts. In the duodenum, there is a positive effect and the higher villi height can justify the higher activity of maltase. In the ileum, the longest trait of the small intestine, there is a negative effect as the villi height was decreased as well as the villi to crypt ratio and the activity of the IAP indicating a detriment of the absorption surface (Han et al., 2012), but a positive effect on the activities of the disaccharase enzymes (maltase and sucrase). The brush-border maltase and sucrase represent the last step in the small intestinal digestion of the starch and their increased activity indicated an improved utilization of the nutrients (Han et al., 2012). Moreover, it has been shown that the ileum plays a significant role in the starch digestion and absorption in fast growing broiler-chickens (Svihus et al., 2004; Zimonja et al., 2009). In addition, the activity of the IAP was higher in the jejunum and ileum of the layers fed the control diet. The IAP acts suppressing the inflammatory responses from the host that may be induced by the lipopolysaccharides from the commensal bacteria (Vaishnava and Hooper, 2007) and promotes several beneficial effects to the intestinal health, including the regulation of calcium absorption and the modulation of the intestinal bacterial growth (Alam et al., 2014; Brun et al., 2014; Malo et al., 2014). This enzyme is considered an excellent marker for the crypt–villus differentiation in chicken (Sabatakou et al., 2007).

Overall, it is not easy to compare our results with the other findings as the effect of the dietary treatments on the intestinal enzymatic activity in poultry has been reported only for the broiler and only for post-hatch chicks (Iji et al., 2001).

In the ileum, there is also a reduction of the γ-GT activity of the layers fed HILM in agreement with a significant decrease of the ileum villi height. The γ-glutamyl transferase is a brush border enzyme which, in the mouse, exhibited a low activity in the crypt and increasing activity up the villus; this activity was stimulated by the dietary protein (Ferraris et al., 1992). This enzyme is involved in the

amino acid transport in the intestine (Smith et al., 1991; Cotgreave and Schuppe-Koistinen, 1994). Thus, the lowest value of γ-GT recorded in the ileum of the HILM group is consistent with the lower protein availability for digestion observed here.

In the literature, the feed restriction, and thus the reduction of feed intake, is reported to lead a reduction of some digestive enzymes such as trypsin (Palo et al., 1995), carboxypeptidase A (Susbilla et al., 2003) but also sucrase (Pinheiro et al., 2004). However, all the above-cited studies are made on young chickens (up to 42 days of age) and the effects can be ascribed to a decrease in the digestive organ mass which induces a similar reduction of the digestive activity. In the present trials, the hens reached the body maturity and thus the effect of feed intake on intestinal enzymatic activity could be less important. However, other studies need to better clarify the effect of feeding insect on the brush border enzyme activity of laying hens.

Conclusion

The use of *H. illucens* larvae meal as total replacement of soybean meal in the laying hens diet from 24 to 45 weeks of age had a negative effect on the feed intake and nutrient digestibility, in particular of the protein, reducing the live weight of the birds at the end of the trial. Probably, the enzymatic activities in the small intestine and of the volatile fatty acid production in the caeca were modified due to the dietary inclusion of the insect meal and these changes were positive in terms of the enhanced production of butyric acid in the caeca, or negative as the reduction of some enzymatic activities in the ileum.

Further studies are needed to find out the optimal level of the dietary inclusion of *H. illucens* meal to balance the negative effects on the feed intake and nutrient digestibility and the positive effects on the digestive system the laying hens.

Acknowledgements. The authors thank the owners and the staff of Casetta Rossa (Presenzano, Caserta, Italy) for making possible the trial.

Author's contributions: MIC participated in the design of the study and in the manuscript writing. MM performed brush border membrane enzymes analysis and participated in the manuscript writing. TF performed brush border membrane enzymes analysis and participated in the manuscript writing. RB carried out intestinal morphometry. OI carried out intestinal morphometry and participated in the manuscript writing. GL supplied insect meals and helped to draft the manuscript. LR carried out intestinal measurements, volatile fatty acid and apparent ileal digestibility coefficients measurements. FB conceived the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

References

Agunbiade, J.A., Adeyemi, O.A., Ashiru, O.M., Awojobi, H.A., Taiwo, A.A., Oke, D.B., Adekunmisi, A.A., 2007. Replacement of fish meal with maggot meal in cassava-based layers' diets. J. Poult. Sci. 44, 278-282.

Alam, S.N., Yammine, H., Moaven, O., Ahmed, R., Moss, A.K., Biswas, B., Muhammad, N., Biswas, R., Raychowdhury, A., Kaliannan, K., Ghosh, S., Ray, M., Hamarneh, S.R., Barua, S., Malo, N.S., Bhan, A.K., Malo, M.S., Hodin, R.A., 2014. Intestinal alkaline phosphatase prevents antibiotic-induced susceptibility to enteric pathogens. Ann. Surg. 259, 715–722.

AOAC, Association of Official Analytical Chemists. 2004. Official Methods of Analysis. 18th edn. AOAC Int., Arlington, VA.

Ballitoc, D.A., Sungo, S., 2013. Ground yellow mealworms (Tenebrio molitor L.) feed supplementation improves growth performance and carcass yield characteristics in broilers. Open Sci. Reposit. Agric. http://dx.doi.org/10.7392/openaccess.23050425.

Barnes, E.M., 1979. The Intestinal Microflora of Poultry and Game Birds During Life and After Storage. J. Appl. Bacteriol. 46, 407-419.

Biasato, I., De Marco, M., Rotolo, L., Renna, M., Dabbou, S., Capucchio, M.T., Biasibetti, E., Tarantola, M., Costa, P., Gai, F., Pozzo, L., Dezzutto, D., Bergagna, S., Gasco L., Schiavone, A., 2016. Effects of dietary Tenebrio molitor meal inclusion in free-range chickens. J. Anim. Physiol. Anim. Nutr. 100, 1104-1112.

Bovera, F., Loponte, R., Marono, S., Piccolo, G., Parisi, G., Iaconisi, V., Gasco, L., Nizza, A., 2016. Use of Tenebrio molitor larvae meal as protein source in broiler diet: Effect on growth performance, nutrient digestibility, and carcass and meat traits. J. Anim. Sci. 94, 639-647.

Bovera, F., Marono, S., Di Meo, C., Piccolo, G., Iannaccone, F., Nizza, A., 2010. Effect of mannanoligosaccharides supplementation on caecal microbial activity of rabbits. Animal 4, 1522–1527.

Bovera, F., Piccolo, G., Gasco, L., Marono, S., Loponte, R., Vassalotti, G., Mastellone, V.,

Lombardi, P., Attia, Y.A., Nizza, A., 2015. Yellow mealworm larvae (*Tenebrio molitor*, L.) as a possible alternative to soybean meal in broiler diets. Br. Poult. Sci. 56, 569-575.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Annal. Biochem. 72, 248-54.

Brun, L.R., Brance, M.L., Lombarte, M., Lupo, M., Di Loreto, V.E., Rigalli, A., 2014. Regulation of intestinal calcium absorption by luminal calcium content: role of intestinal alkaline phosphatase. Mol. Nutr. Food. Res. 58, 1546–1551.

Cotgreave, I.A0, Schuppe-Koistinen, I., 1994. A role for gamma-glutamyl transpeptidase in the transport of cystine into human endothelial cells: relationship to intracellular glutathione. Biochem. Biophys. Acta 1222, 375–382.

De Marco, M., Martínez, S., Hernandez, F., Madrid, J., Gai, F., Rotolo, L., Belforti, M., Bergero, D., Katz, H., Dabbou, S., Kovitvadhi, A., Zoccarato, I., Gasco, L., Schiavone, A., 2015. Nutritional value of two insect larval meals (*Tenebrio molitor* and *Hermetia illucens*) for broiler chickens: Apparent nutrient digestibility, apparent ileal amino acid digestibility and apparent metabolizable energy. Anim. Feed Sci. Technol. 209, 211–218.

Esmail, S.H., 2013. Factors affecting feed intake of chickens. World Poult. Sci. 29, 15-17.

Ferraris, R.P., Villenas, S.A., Diamond, J., 1992. Regulation of brush border enzyme activities and enterocyte migration rates in mouse small intestine. Am. J. Physiol. 262, G1047–G1059.

Finke, M.D., 2007. Estimate of chitin in raw whole insects. Zoo Biol. 26, 105–115.

Han, J., Miranda-Saavedra, D., Luebbering, N., Singh, A., Sibbet, G., Ferguson, M.A., Cleghon, V., 2012. Deep evolutionary conservation of an intramolecular protein kinase activation mechanism. PLoS One 7, e29702. doi: 10.1371/journal.pone.0029702.

Iji, P.A., Saki, A., Tivey, D.R., 2001. Body and intestinal growth of broiler chicks on a commercial starter diet. 2. Development and characteristics of intestinal enzymes. Br. Poult. Sci. 42, 514-522.

Khempaka, S., Chitsatchapong, C., Molee, W., 2011. Effect of chitin and protein constituents in

shrimp head meal on growth performance, nutrient digestibility, intestinal microbial populations, volatile fatty acids, and ammonia production in broilers. J. Appl. Poult. Res. 20, 1–11.

Lohmann Brown classic Management Guide, 2011; http://www.specialisedbreeders.com.au/wp-content/uploads/2016/04/LTZ-Management-Guide-LB-Classic-EN.pdf. Accessed 04/12/2016.

Longvah, T., Mangthya, K., Ramulu, P., 2011. Nutrient composition and protein quality evaluation of eri silkworm (Samia ricinii) prepupae and pupae. Food Chem. 128, 400–403.

Loponte, R., Marono, S., Iaconisi, V., Piccolo, G., Parisi, G., Bovera, F., 2016. Caecal volatile fatty acid production of broilers fed Tenebrio molitor larvae meal. In:INSECTA 2016 International Symposium on Insects as Food, Feed and Non-food, Magdeburg (Germany), 12th September.

Loponte, R., Nizza, S., Bovera, F., De Riu, N., Fliegerova, K., Lombardi, P., Vassalotti, G., Mastellone, V., Nizza, A., Moniello, G., 2017. Growth performance, blood profiles and carcass traits of Barbary partridge (Alectoris barbara) fed two di□erent insect larvae meals (Tenebrio molitor and Hermetia illucens). Res. Vet. Sci. 115, 183-188.

Mahdavi, R., Torki, M., 2009. Study on usage period of dietary protected butyric acid on performance, carcass characteristics, serum metabolite levels and humoral immune response of broiler chickens. J. Anim .Vet. Adv. 8, 1702–1709.

Makkar, H.P.S., Tran, G., Heuze, V., Ankers, P., 2014. State-of-the-art on use of insects as animal feed. Anim. Feed Sci. Technol. 197, 1-33.

Malo, M.S., Moaven, O., Muhammad, N., Biswas, B., Alam, S.N., Economopoulos, K.P., Gul, S.S., Hamarneh, S.R., Malo, N.S., Teshager, A., Mohamed, M.M., Tao, Q., Narisawa, S., Millan, J.L., Hohmann, E.L., Warren, H.S., Robsonm S.C., Hodinm R.A., 2014. Intestinal alkaline phosphatase promotes gut bacterial growth by reducing the concentration of luminal nucleotide triphosphates. Am. J. Physiol. Gastrointest. Liver Physiol. 306, G826–G838.

Marono, S., Loponte, R., Lombardi, P., Vassalotti, G., Pero, M.E., Russo, F., Gasco, L., Parisi, G., Piccolo, G., Nizza, S., Di Meo, C., Attia, Y.A., Bovera, F., 2017. Productive performance and blood profiles of laying hens fed Hermetia illucens larvae meal as total replacement of soybean

meal from 24 to 45 week of age. Poult. Sci. 96, 1783-1790.

Marono, S., Piccolo, G., Loponte, R., Di Meo, C., Attia, Y.A., Nizza, A., Bovera, F., 2015. In vitro crude protein digestibility of Tenebrio molitor and Hermetia illucens insect meals and its correlation with chemical composition traits. It. J. Anim. Sci. 14, 338-343.

Maurer, V., Holinger, M., Amsler, Z., Früh, B., Wohlfahrt, J., Stamer, A., Leiber, F., 2016. Replacement of soybean cake by Hermetia illucens meal in diets for layers. J. Insects Food and Feed 2, 89-9014.

Montagne, L., Pluske, J.R., Hampson, D.J., 2003. A review of interactions between dietary fibre and the intestinal muco.sa, and their consequences on digestive health in young non-ruminant animals. Anim. Feed Sci. Technol. 108, 95–117.

Mroz, Z., 2005. Organic acids as potential alternatives to antibiotic growth promoters for pigs. In: R. O. Ball and R. T. Zijlstra, editors, Advance in pork production. Vol. 16. University of Alberta Department of Agricultural, Food and Nutritional Science, Edmonton, AB, Canada, 169–182.

National Research Council, 1994. Nutrient requirements of poultry, 9th revised edition. Washington, DC, National Academy Press.

Nisbet, D.J., Corrier, D.E., Ricke, S.C., Hume, M.E., Byrd, J.A., DeLoach, J.R., 1996. Maintenance of the biological efficacy in chicks of a cecal competitive exclusion culture against Salmonella by continuous-flow fermentation. J. Food Prot. 59, 1270–1283.

Oonincx, D.G.A.B., Van Itterbeeck, J., Heetkamp, M.J.W., Van den Brand, H., van Loon, J.J.A., van Huis, A., 2010. An exploration on greenhouse gas and ammonia production by insect species suitable for animal or human consumption. PloS One 5, doi: 10.1371/journal.pone.0014445.

Oyegoke, O.O., Akintola, A.J., Fasoranti, J.O., 2006. Dietary potentials of the edible larvae of Cirina forda (westwood) as a poultry feed. Afr. J. Biotechnol. 5, 1799-1802.

Pal, P., Roy, S., 2014. Edible insects: Future of human food - A Review. Int. Letters Nat. Sci. 26, 1-11.

Palo, P.E., Sell, J.L., Piquer, F.J., Soto-Salanova, M.F., Vilaseca, L., 1995. Effect of early nutrient

restriction on broiler chickens. 1. Performance and development of the gastrointestinal tract. Poult. Sci. 74, 88-101.

Pinheiro, D.F., Cruz, V.C., Sartori, J.R., Vicentini-Paulino, M.L.M., 2004. Effect of early feed restriction and enzyme supplementation on digestive enzyme activities in broilers. Poult. Sci. 83, 1544-1550.

Sabatakou, O., Paraskevakou, E., Tseleni-Balafouta, S., Patsouris, E., 2007. Histochemical study of alkaline phosphatase activity in the chicken intestine. Bulg. J. Vet. Med. 10, 83−93.

Saemann, M.D., Bohmig, G.A., Osterreicher, C.H., Burtscher, H., Parolini, O., Diakos, C., Stock, J., Hor, W.H., Zlabinger, J.G., 2000. Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. The Faseb J. 14, 2380-2382.

SAS, Statistical Analyses System, 2000. SAS Institute Inc., SAS/ STAT Software, Version 9, Cary, NC, SAS Institute Inc.

Shirazy-Beechey, S.P., Hirayama, B.A., Wang, Y., Scott, D., Smith, M.W., Wright, E.M., 1991. Ontogenic development of lamb intestinal sodium glucose co-transporter is regulated by diet. J. Physiol. 437, 699-708.

Smith, T.K., Gibson, C.L., Howlin, B.J., Pratt, J.M., 1991. Active transport of amino acids by gamma-glutamyl transpeptidase through Caco-2 cell monolayers. Biochem. Biophys. Res. Commun. 178, 1028–1035.

Stanco, G., Di Meo, C., Calabrò, S., Nizza, A., 2003. Prediction of nutritive value of diets for rabbits using an in vitro gas production technique. World Rabbit Sci. 11, 199-210.

Susbilla, J.P., Tarvid, I., Gow, C.B., Frankel, T.L., 2003. Quantitative feed restriction or meal-feeding of broiler chicks alter functional development of enzymes for protein digestion. Br. Poult. Sci. 44, 698-709.

Svihus, B., Juvik, E., Hetland, H., 2004. Causes for improvement in nutritive value of broiler chicken diets with whole wheat instead of ground wheat. Br. Poult. Sci. 45, 55-60.

Tibaldi, E., Hakim, Y., Uni, Z., Tulli, F., de Francesco, M., Luzzana, U., Harpaz, S., 2006. Effects of the partial substitution of dietary fish meal by differently processed soybean meals on growth performance, nutrient digestibility and activity of intestinal brush border enzymes in the European sea bass (Dicentrarchus labrax). Aquaculture 261, 182–193.

Topping, D.L., Clifton, P.M., 2001. Short-chain fatty acids and human colonic function: Roles of resistant starch and nonstarch polysaccharides. Physiol. Rev. 81, 1031–1064.

Uni, Z., Ganot, S., Sklan, D., 1998. Posthatched development of mucosal function in the broiler small intestine. Poult Sci. 77, 75-82.

Uni, Z., Noy, Y., Sklan, D., 1996. Developmental parameters of the small intestines in heavy and light strain chicks preand post-hatch. Br. Poult. Sci. 36, 63–71.

Uni, Z., Noy, Y., Sklan, D., 1995. Post hatch changes in morphology and function of the small intestines in heavy and light strain chicks. Poult. Sci. 74, 1622–9.

Vaishnava, S., Hooper, L.V., 2007. Alkaline phosphatase: keeping the peace at the gut epithelial surface. Cell Host Microbe 2, 365–67.

van Der Wielen, P.W., Biesterveld, S., Notermans, S., Hofstra, H., Urlings, B.A., van Knapen, F., 2000. Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth. Appl. Environ. Microbiol. 66, 2536-2540.

Vogtmann, H., Frirter, P., Prabuck, L., 1975. A new method of determining metabolizability of energy and digestibility of fatty acids in broiler diets. Br. Poult. Sci. 16, 531–534.

Wang, Y.C., Chen, Y.T., Li, X.R., Xia, J.M., Du, Q.S., Zhi, C., 1996. Study on rearing the larvae of Tenebrio molitor L. and the effects of its processing and utilization. Acta Agric. Univ. Henanensis 30, 288–92.

Zimonja, O., Svihus, B., 2009. Effects of processing of wheat or oats starch on physical pellet quality and nutritional value for broilers. Anim. Feed Sci. Technol. 149, 287-297.

Table 1. Proximate composition, mineral and essential amino acid composition (% as fed) of the *Hermetia illucens* larvae meal and soybean meal.

	Hermetia illucents larvae meal	Soybean meal
Proximate composition		
Dry matter	97.8	90.0
Crude protein	61.3	43.4
Ether extract	4.6	1.1
ADF	12.1	5.9
ADF-linked protein	5.59	1.78
Ash	7.8	6.0
Mineral composition		
Ca[1]	6.90	2.83
Total P[1]	0.91	0.57
Na[1]	0.12	0.16
Essential Amino Acid composition		
Lysine[1]	4.05	2.92
Methionine[1]	1.30	0.61
Methionine+Cystine[1]	1.42	1.33
Isoleucine[1]	3.11	2.30
Tryptophan[1]	0.30	0.73
Valine[1]	5.02	2.11
Threonine[1]	2.32	1.74

[1] obtained by producers

Table 2. Ingredient composition and proximate analysis of the test diets.

	HIML	SBM
Ingredient composition (g/kg)		
Maize grain	653.0	583.0
Soybean meal	-	235.0
Insect meal	170.0	-
$CaCO_3$ grains	80.0	80.0
Dehulled sunflower meal	50.0	50.0
Vegetable oil	10.0	15.0
Mineral and Vitamin supplement[1]	30.0	30.0
Monocalcium phosphate	5.00	5.00
Salt	2.00	2.00
Proximate analysis (% as feed) and energy content (Kcal/kg)		
Dry matter[2]	90.5	90.1
Crude protein[2]	17.9	18.1
Crude fiber[2]	4.1	4.0
Ether extract[2]	4.3	4.3
ADF[2]	3.8	3.5
ADF linked protein[2]	2.88	1.52
Ash[2]	14.2	14.2
NDF[2]	15.2	14.0
Metabolizable Energy[3]	2,745	2,780
Mineral and EAA content (% as feed)		
Ca[3]	4.96	4.26
Total P[3]	0.67	0.69

Na[3]	0.30	0.19
Lysine[3]	0.91	0.90
Methionine[3]	0.64	0.55
Methionine+Cystine[3]	0.86	0.84
Isoleucine[3]	0.79	0.77
Tryptophan[3]	0.16	0.21
Valine[3]	1.19	0.82
Threonine[3]	0.64	0.63

HILM: Hermetia illucens larvae meal; SBM: soybean meal

[1] Provided 20 g of celite and 10 g of mineral and vitamin supplements. Per kilogram: vitamin A (retinyl acetate) 20,000 IU, vitamin D3 (cholecalciferol) 6,000 IU, vitamin E (dl-α-tocopheryl acetate) 80 IU, vitamin B1(thiamine monophosphate) 3 mg, vitamin B2 (riboflavin) 12 mg, vitamin B6 (pyridoxine hydrochloride) 8 mg, vitamin B12 (cyanocobalamin) 0.04 mg, vitamin K3 (menadione) 4.8 mg; vitamin H (d biotin) 0.2 mg, vitamin PP (nicotinic acid) 48 mg, folic acid 2 mg, calcium pantothenate 20 mg, manganous oxide 200 mg, ferrous carbonate 80 mg, cupric sulphate pentahydrate 20 mg, zinc oxide 120 mg, basic carbonate monohydrate 0.4 mg, anhydrous calcium iodate 2 mg, sodium selenite 0.4 mg, choline cloride 800 mg, 4-6-phitase 1,800 FYT, D.L. methionine 2,600 mg, canthaxanthin 8 mg.

[2] analysed composition

[3] calculated composition

Table 3. Weight and length of the intestine of hens fed the test diets over 21 weeks (n = 18 per group)

	HIML	SBM	RMSE	P value
Live weight, kg	1.89[b]	2.10[a]	0.154	0.0121
	Intestine weight, % LW			
Full digestive tract	10.45[a]	8.81[b]	0.68	0.0330
Empty digestive tract	6.42	5.76	0.57	0.1263
Crop	0.30	0.24	0.14	0.1116
Duodenum	1.08	1.14	0.16	0.3723
Jejunum	1.21	1.11	0.18	0.2874
Ileum	1.14	1.21	0.23	0.5758
Caeca	0.71	0.72	0.09	0.7260
Colon	0.43	0.36	0.12	0.2267
Cloaca	1.45	1.04	0.60	0.1739
	Intestine lenght, % LW			
Entire intestinal tract	10.09	10.13	0.71	0.5698
Duodenum	1.66	1.87	0.66	0.5227
Jejunum	2.74	2.63	0.76	0.7763
Ileum	3.26	3.24	0.71	0.9395
Caeca	1.44	1.50	0.25	0.6012
Colon	0.59	0.54	0.11	0.4522
Cloaca	0.35	0.34	0.13	0.8760

HILM: Hermetia illucens larvae meal; SBM: soybean meal; RMSE: root mean square error; LW: live weight.

a, b: $P < 0.05$

Table 4. Specific activity (UI) of maltase, saccarase, intestine alkaline phosphatase (IAP), ɤ-glutamytransferase (ɤGT) measured in different digestive tracts of hens fed test diets over 21 weeks (n = 18 per group)

	HIML	SBM	RMSE	P value
Duodenum				
Maltase	9.86[a]	7.13[b]	2.55	0.044
Saccarase	1.71	1.83	0.57	0.679
IAP	6.83	8.19	2.97	0.360
ɤGT	0.205	0.184	1.91	0.492
Jejunum				
Maltase	8.93	6.41	3.14	0.129
Saccarase	3.22	2.30	1.54	0.252
IAP	5.40[b]	8.30[a]	2.49	0.036
ɤGT	0.198	0.229	2.61	0.466
Ileum				
Maltase	11.21[a]	5.20[b]	3.98	0.010
Saccarase	2.35[A]	1.40[B]	0.57	0.005
IAP	3.57[b]	5.20[a]	1.29	0.026
ɤGT	0.107[B]	0.188[A]	1.21	0.001

HILM: Hermetia illucens larvae meal; SBM: soybean meal; RMSE: root mean square error.

A, B: $P < 0.01$; a, b: $P < 0.05$

Table 5. Intestinal morphometry of small intestine of hens fed the test diets over 21 weeks (n = 18 per group)

	HIML	SBM	RMSE	P value
Duodenum				
Villi height	934.2[a]	828.5[b]	105.4	0.0321
Crypt depth	153.7	157.8	25.56	0.6240
Villi/Crypt	6.07	5.25	0.67	0.0895
Jejunum				
Villi height	809.0[b]	946.3[a]	133.5	0.0294
Crypt depth	184.6	173.4	37.39	0.5494
Villi/Crypt	4.38	5.46	0.76	0.1204
Ileum				
Villi height	916.4[b]	1055.7[a]	91.30	0.0184
Crypt depth	205.4[a]	176.6[b]	17.23	0.0329
Villi/Crypt	4.45[b]	5.98[a]	0.29	0.0236

HILM: Hermetia illucens larvae meal; SBM: soybean meal; RMSE: root mean square error.

a, b: $P < 0.05$

Table 6. Volatile fatty acid production in the caecal content of hens fed the test diets over 21 weeks (n = 18 per group)

	HIML	SBM	RMSE	P value
	Absolute values, mmol/l			
Acetate	50.68[a]	37.25[b]	11.44	0.0290
Propionate	13.83	10.25	5.24	0.1802
Butyrate	6.86[A]	4.22[B]	1.47	0.0022
Isobutyrate	0.71	0.77	0.21	0.6541
Valerianic	1.50	1.22	0.46	0.2238
Isovalerianic	1.06	0.89	0.25	0.3832
Total VFA	74.59[a]	54.52[b]	17.38	0.0312
	Relative values, % of total VFA			
Acetate	68.50	68.22	5.07	0.9128
Propionate	17.92	18.51	4.27	0.7782
Butyrate	9.26	7.98	1.63	0.1276
Isobutyrate	0.96[B]	1.45[A]	0.27	0.0033
Valerianic	1.99	2.28	0.42	0.1764
Isovalerianic	1.38	1.72	0.47	0.1500

HILM: Hermetia illucens larvae meal; SBM: soybean meal; RMSE: root mean square error; VFA: volatile fatty acids

A, B: $P < 0.01$; a, b: $P < 0.05$

Table 7. Coefficients of apparent ileal digestibility (%) of hens fed the test diets over 21 weeks (n = 9 per group)

	HIML	SBM	RMSE	P value
Dry matter	67.63[b]	70.27[a]	2.36	0.0421
Organic matter	69.17[b]	72.18[a]	2.24	0.0177
Protein	67.67[B]	78.29[A]	1.84	<0.0001
Lipid	73.01	77.34	5.03	0.1071

HILM: Hermetia illucens larvae meal; SBM: soybean meal; RMSE: root mean square error

A, B: $P < 0.01$; a, b: $P < 0.05$

No figures are in the manuscript

Highlights

- The effect of Hermetia illucens on intestinal morphometry and activity of laying hens was studied.
- The insect meal decreased digestibility and modified enzymatic activity in the small intestine.
- Dietary inclusion of insect meal enhanced the production of butyric acid in the caeca.