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I. Biasato[1], M. De Marco[1], L. Rotolo[2], M. Renna[2], C. Lussiana[2], S. Dabbou[2], M.T. Capucchio[1], E. Biasibetti[1], P. Costa[1], F. Gai[3], L. Pozzo[3], D. Dezzutto[4], S. Bergagna[4], S. Martínez[5], M. Tarantola[1,6], L. Gasco[2,3], and A. Schiavone[1,6]

[1]Dipartimento di Scienze Veterinarie, Università degli Studi di Torino, Italy

[2]Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università degli Studi di Torino, Italy

[3]Istituto di Scienze delle Produzioni Alimentari (ISPA), CNR, Italy

[4]Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Torino, Italy.

[5]Departamento de Producción Animal, Universidad de Murcia, Spain

[6]Istituto di Ricerche Interdisciplinari sulla Sostenibilità, Università degli Studi di Torino, Italy

Correspondence: Prof. Achille Schiavone, Dipartimento di Scienze Veterinarie, Università degli Studi di Torino, largo Paolo Braccini 2, 10095 Grugliasco (TO), Italy. Tel: +39-011-6709208; Fax: +39-011-6709240; E-mail: achille.schiavone@unito.it

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Summary

Insects are currently being considered as a novel protein source for animal feeds, because they contain a large amount of protein. The larvae of *Tenebrio molitor* (TM) have been shown to be an acceptable protein source for broiler chickens in terms of growth performance, but till now no data on histological or intestinal morphometric features have been reported. The present study has had the aim of evaluating the effects of dietary TM inclusion on the performance, welfare, intestinal morphology and histological features of free-range chickens. A total of 140 medium-growing hybrid female chickens were free-range reared and randomly allotted to two dietary treatments: i) a control group and ii) a TM group, in which TM meal was included at 75 g/kg. Each group consisted of five pens as replicates, with 14 chicks per pen. Growth performance, hematological and serum parameters and welfare indicators were evaluated and the animals were slaughtered at the age of 97 days. Two birds per pen (10 birds/treatment) were submitted to histological (liver, spleen, thymus, bursa of Fabricius, kidney, heart, glandular stomach and gut) and morphometric (duodenum, jejunum and ileum) investigations. The inclusion of TM did not affect the growth performance, hematological or serum parameters. The morphometric and histological features were not significantly affected either, thus suggesting no influence on nutrient metabolization, performance or animal health. Glandular stomach alterations (chronic flogosis with epithelial squamous metaplasia) were considered paraphysiological in relation to free-range farming. The observed chronic intestinal flogosis, with concomitant activation of the lymphoid tissue, was probably due to previous parasitic infections, which are very frequently detected in free-range chickens. In conclusion, the findings of this study show that yellow mealworm inclusion does not affect the welfare, productive performances or morphological features of free-range chickens, thus confirming that TM can be used safely in poultry diets.

Keywords

Poultry, insect meal, yellow mealworm, histology, morphometry.

Introduction

Insects are currently considered a novel protein source for animal feeds (Sánchez-Muros et al., 2014). Insect meals exhibit a great potential for becoming a standard ingredient in animal feeding, because of the high quality and quantity of protein (Ramos-Elorduy, 1997). Furthermore, the low competitiveness with human food (Ballitoc and Sun, 2013) and the reduction in environmental impact in terms of energy cost, land area utilization and footprints (Oonincx and de Boer, 2012; Makkar et al., 2014; Sánchez-Muros et al., 2014), make insects promising in an ecological perspective. Invertebrates are included in the European Union Feed Material Register as a feed material, even though they are currently only authorized for pets. However, insect-derived feeds could also represent a possible ingredient for livestock animals, such as poultry, pigs and fish (Veldkamp et al., 2012; Van Huis, 2013; Makkar et al., 2014; Henry et al., 2015). In particular, the most promising insect species for industrial production are *Hermetia illucens* (black soldier fly), *Musca domestica* (common house fly), *Tenebrio molitor* (yellow mealworm), *Bombyx mori* (silkworm) and several grasshoppers (Van Huis, 2013).

Considering that insects are consumed naturally by wild birds and free-range poultry (Zuidhof et al., 2003), some studies have evaluated the feasibility of using insects as an alternative feed source for poultry (Khatun et al., 2003; Wang et al., 2005; Oyegoke et al., 2006; Adenjii, 2007; Hwangbo et al., 2009; Ijaiya and Eko, 2009; Ballitoc and Sun, 2013). Some authors have observed no differences in growth performance (in terms of feed intake, body weight gain and feed conversion efficiency) in broilers fed a control diet and an insect-based diet (Wang et al., 2005; Oyegoke et al., 2006; Adenjii, 2007; Ijaiya and Eko, 2009). Other studies have reported that insect meal inclusion in chicken diets improved animal growth indexes (Khatun et al., 2003; Hwangbo et al., 2009; Ballitoc and Sun, 2013). The same studies also observed an improvement in carcass yield characteristics, such as dressing percentage, breast

muscle, thigh muscle, slaughter, dressed carcass and eviscerated weights (Khatun et al., 2003; Hwangbo et al., 2009; Ballitoc and Sun, 2103).

The larvae of *Tenebrio molitor* (TM) are easily bred, because of their efficient growth on dried and cooked waste materials from fruit, vegetables and cereals in various combinations. For this reason, they are already industrially produced as feeds for pets and zoo animals, including birds, reptiles, small mammals, amphibians and fish (Makkar et al., 2014). On a dry matter basis, the meal derived from TM larvae contains a large amount of crude protein (440 to 690 g/kg) and fat (230 to 470 g/kg) (Veldkamp et al., 2012). In livestock, TM has been shown to be an acceptable protein source for broiler chickens (Ramos-Elorduy et al., 2006; Ballitoc and Sun, 2013) and fish (Roncarati et al., 2015; Belforti et al., in press).

Intestinal morphology is the main indicator of gut health and functioning (Kristy et al., 2005). Dietary protein level and digestibility have been reported to significantly affect the intestinal development and the mucosal architecture of the gastrointestinal tract of broilers (Laudadio et al., 2012; Qaisrani et al., 2014). Intestinal development can be assessed through morphometric measurements of the villus height (to determine the area available for digestion and absorption) and crypt depth (the region in which new intestinal cells are formed) (Franco et al., 2006). The villus height/crypt depth ratio can also be evaluated, because it generally gives an indication of the likely maturity and functional capacity of the enterocytes (Hampson, 1986).

The nutrient profile and structure of protein sources may also vary significantly, and thus have different effects on nutrient utilization and metabolism, and consequently on some blood constituents, such as serum cholesterol, triglycerides and uric acid (Wang et al., 2015). Some blood parameters have been used as physiological indicators of the stress response of chickens. The heterophils-to-lymphocytes (H/L) ratio is affected by stress factors and could be used as an indicator of stress in poultry (De Marco et al., 2013; Salamano et al., 2010). The

H/L ratio is correlated to a bird's health status and responds to stimuli associated with diet, chronic bacterial infections, stress, light and trauma, and it varies according to the change in the percentage of heterophils and lymphocytes in the blood (Gross and Siegel, 1983; Maxwell, 1993; Maxwell and Robertson, 1998). Finally, diet composition can also affect the litter quality (Lynn and Elson, 1990; Jones et al., 2005), which is directly related to the development of footpad dermatitis (FPD) (Bessei, 2006). FPD is a condition that involves inflammation and necrotic lesions on the plantar surface of the bird's feet and is considered to be an important welfare indicator in broilers (Ekstrand et al., 1997; Meluzzi et al., 2008; Welfare Quality®, 2009).

Although insect meals are considered a suitable ingredient for poultry feeding (Veldkamp et al., 2012; Van Huis, 2013; Makkar et al., 2014), there is currently a lack of data on their utilization. Apart from the evaluation of growth performance and carcass yield characteristics, no anatomopathological or morphometric investigations, blood traits analysis or welfare assessments have been carried out on chickens fed diets with insect meal inclusion. Therefore, the aim of the present study was to evaluate the effects of TM dietary inclusion on the productive performance, intestinal morphology, histological features, hematochemical parameters and welfare of free-range chickens.

Materials and Methods

The study was performed by the Department of Veterinary Sciences and the Department of Agricultural, Forest and Food Sciences of the University of Torino (Italy) in collaboration with an external farm called "Fattoria La Fornace", located in Montechiaro d'Asti (At - Italy). The experimental protocol was designed according to the guidelines of the current European and Italian laws on the care and use of experimental animals (Directive 2010/63/EU, put into

force in Italy with D.L. 2014/26). The experiment was carried out between November-December 2014, when the photoperiod was 9-10L:14-15D.

Birds and diets

In this experiment, a total of 140 female Label Hubbard hybrid chickens (female: JA 57 × male: S77CN), a medium-growing genotype, were used. All the birds were free-range reared, in identical environmental conditions, throughout the experimental trial. At the age of 43 days (average weight 715 g), the birds were randomly allotted to two dietary treatments, each consisting of five pens as replicates, with 14 chicks per pen. Each pen had an indoor area (2.5×3.5 m) and an outdoor paddock of the same dimension (2.5×3.5 m). The floor was covered, to a height of 10 cm, with wood shaving litter.. The birds were only exposed to natural light. Two diets were formulated: a control diet (C), normally used by the breeder, and an experimental diet (TM), in which TM meal (Gaobeidian Shannong Biology Co. Ltd., Gaobeidian, Hebei province - China) was included at 75 g/kg in substitution of corn gluten meal (Table 1). The diets were designed to meet or exceed the current poultry requirements (NRC, 1994). Both diets were isonitrogenous and isoenergetic and were formulated using the apparent metabolizable energy (AMEn) values for TM calculated for broiler chickens (De Marco et al., 2015). Feed and water were provided *ad libitum*.

The experiment lasted 54 days. The average chicken weight and feed intake were recorded at the beginning and at the end of the experiment on a pen basis. The final body weight was recorded on day 97, and the feed conversion ratio was calculated for the 43-97 day period.

The chicks were vaccinated at hatching against coccidiosis, Newcastle disease and infectious bronchitis. All the birds were individually identified with a shank ring.

Chemical analyses of the diets

151 The diets were subsequently ground to pass through a 0.5-mm sieve and stored in airtight
152 plastic containers for DM, ash, CP, crude fibre (AOAC, 2005) and EE (Folch et al., 1957)
153 determination. The fatty acid composition of the control and TM diets was assessed using the
154 method described by Alves *et al.* (2008). Fatty acid methyl esters were separated, identified
155 and quantified on the basis of the chromatographic conditions reported by Renna *et al.* (2014).
156 Heptadecanoic acid (C17:0) was used as the internal standard. The results were expressed in
157 absolute values as g/kg DM.
158 In order to perform the AA determination, samples of the diets were prepared using a 22 h
159 hydrolysis step in 6 HCl at 112ºC under a nitrogen atmosphere. Performic acid oxidation
160 occurred prior to acid hydrolysis for methionine and cystine. The AA in the hydrolysate was
161 determined by means of HPLC after postcolumn derivatization, according to the procedure
162 described by Madrid et al. (2013). Tryptophan was not determined.
163 All the analyses were performed in duplicate.
164
165 *Hematological and serum parameters*
166 At the end of the experiment (day 97), blood samples were collected at slaughtering from 4
167 birds per pen: 2.5 mL was placed in an EDTA tube and 2.5 mL in a serum-separating tube. A
168 blood smear was prepared, using one glass slide for each bird, from a drop of blood without
169 anticoagulant. The smears were stained using May-Grünwald and Giemsa stains (Campbell,
170 1995). The total red and white blood cell counts were determined in an improved Neubauer
171 haemocytometer on blood samples previously treated with a 1:200 Natt-Herrick solution. One
172 hundred leukocytes, including granular (heterophils, eosinophils and basophils) and non-
173 granular (lymphocytes and monocytes) leukocytes, were counted on the slide and the H/L
174 ratio was calculated. The tubes without anticoagulant were left to clot in a standing position at
175 room temperature for approximately two hours to obtain serum. The serum was separated by

means of centrifugation at 700 × g for 15 minutes and frozen at -80°C until analysis. The total proteins were quantified by means of the "biuret method" (Bio Group Medical System kit; Bio Group Medical System, Talamello, 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), triglycerides, cholesterol, glucose, phosphorus, magnesium, iron, uric acid and creatinine serum concentrations were measured by means of enzymatic methods in a clinical chemistry analyzer (Screen Master Touch, Hospitex diagnostics Srl., Florence, Italy).

Slaughtering procedures, footpad dermatitis lesion assessment, histological investigations and morphometric analysis

On day 97, all the chickens were individually marked, weighed and slaughtered in a commercial abattoir. The plucked and eviscerated carcasses were obtained and the head, neck, feet and abdominal fat were removed to obtain carcass-for-grilling. The weight of the breasts, thighs, deboned thighs, spleen, bursa of Fabricius, liver, gizzard and abdominal fat were recorded.

In order to evaluate the FPD lesions, the feet collected at the slaughterhouse were assessed macroscopically using the so-called Swedish footpad scoring system (Ekstrand et al., 1997). According to this system, 0 = no lesion, slight discoloration of the skin or healed lesion; 1= mild lesion, superficial discoloration of the skin and hyperkeratosis; 2 = severe lesion, affected epidermis, blood scabs, hemorrhage and severe swelling of the skin.

After slaughtering, 2 birds per pen (10 birds/treatment) were sampled for anatomopathological investigations. Intestinal segment samples (approximately 5 cm in length) of the duodenum, jejunum, ileum and caecum were excised and flushed with 0.9% saline to remove all the contents. The collected intestine segments were the loop of the

duodenum, the tract before Meckel's diverticulum (jejunum), the tract before the ileocolic junction (ileum) and the apex of the caeca (caecum). Samples of the liver, spleen, thymus, bursa of Fabricius, kidneys, heart and glandular stomach were also collected. The gut segments were fixed in both 10% buffered formalin (for the histological examination) and Carnoy's solution (for the morphometric analysis), while the other organ samples were only fixed in 10% buffered formalin solution. The tissues were routinely embedded in paraffin wax blocks, sectioned at a thickness of 5 µm, mounted onto glass slides and stained with Haematoxylin & Eosin (H&E). Morphometric analyses (Image Pro-Plus software) (Fig. 1A) were performed on 10 well-oriented and intact villi and 10 crypts chosen from the duodenum, jejunum and ileum (Qaisrani et al., 2014). The evaluated morphometric indices were villus height (Vh; from the tip of the villus to the crypt), crypt depth (Cd; from the base of the villi to the submucosa) and their ratio (Vh/Cd) (Laudadio et al., 2012). Histological changes were scored using a semi-quantitative scoring system as follows: absent or minimal (score 0), mild (score 1) and severe (score 2). The semi-quantitative scoring system was assessed evaluating the defined parameters of each organ (Table 2).

Statistical analysis

The statistical analysis was performed using SPSS 17 for Windows (SPSS, Inc., Chicago, IL, USA; SPSS, 2008). The experimental unit was the pen. The influence of diet on the performance parameters, hematological and serum biochemical traits and intestinal morphometric measurements were analyzed using Student's *t*-tests for independent samples. Morphometric data were also analyzed by means of one-way ANOVA (post hoc test: Duncan's multiple range test) to evaluate the influence of the intestinal segment within each dietary treatment. Mann-Whitney *U* tests were used to compare the histological scores between treatments for each considered organ (spleen, thymus, bursa of Fabricius, liver,

glandular stomach, intestine, heart and kidney). Kruskal-Wallis tests were used to compare intestine alterations among the considered segments (duodenum, jejunum, ileum and caecum), within each dietary treatment. The results were considered statistically significant when associated with a lower probability than 5%, and highly significant if the probability was lower than 1%. The results were expressed as mean and pooled standard error of the mean (SEM).

Results

The inclusion of TM in the medium-growing diet in the 43-97d period did not affect the growth and slaughtering performances (Table 3), the blood and serum traits (Table 4) or the FPD lesion incidence. The blood parameters fell within the physiological range.

The morphometric data are summarized in Table 5. There was no significant difference in the morphology of the small intestine between the two dietary treatments ($P > 0.05$). In the C group, the Vh was greater ($P < 0.01$) in the duodenum than in the other gut segments, while the Vh/Cd ratio was only significantly different ($P = 0.01$) between the duodenum and the ileum. In the TM group, Vh and Cd were greater ($P < 0.01$ and $P = 0.03$) in the duodenum than in the other gut segments.

The glandular stomach, intestine, spleen, thymus, bursa of Fabricius and liver were the most frequently affected organs, while the heart and kidneys showed no significant alterations. Chronic inflammation, with lymphoid tissue activation, was observed in the intestinal segments (Fig. 1B). The glandular stomach showed lymphoplasmacytic flogosis, with focal to multifocal lymphoid tissue activation and different degrees of severity of epithelial squamous metaplasia (Fig. 1C-D). The jejunum and the caecum showed the most severe alterations in both dietary treatments ($P < 0.01$). White pulp hyperplasia or depletion was identified in the spleen, while cortical depletion was detected in the thymus. Follicular depletion, with or

without intrafollicular cysts, was observed in the bursa of Fabricius (Fig. 2A-B). Finally, the liver showed different degrees of lymphoid tissue activation (Fig. 2C-D). The histological changes were not significantly different between the dietary treatments ($P > 0.05$).

Discussion

The trial was set up to study the effect of TM inclusion in the diet of medium-growing chickens reared in free-range conditions. The inclusion of TM did not affect the performance, the blood and serum traits or the welfare parameters of the birds. These results confirm that TM can be used safely in poultry diets. Ramos-Elorduy et al. (2006) showed that up to 100 g/kg of dried yellow mealworms can be included in a broiler starter diet based on sorghum and soybean meal, without negative effects on either performance or palatability. Ballitoc and Sun (2013), including different levels of TM meal (5, 10, 20 and 100 g/kg, respectively) in a standard commercial broiler diet, pointed out that the inclusion level of 10 g/kg TM had a great impact on the growth performance of the broilers and improved the final body weight, feed intake and feed efficiency, as well as the slaughter yield. Bovera et al. (2015) showed that 30% inclusion of TM meal in the diet of Shaver brown broilers did not affect the final body weight or blood traits, except for uric acid, albumin/globulin ratio, AST and ALT. In another study, it was noted that TM could be successfully used to replace 4% soyabean meal in laying hens diets (Wang et al., 2015).

FPD is a condition that involves inflammation and necrotic lesions on the plantar surface of the bird's feet. It is considered to be an important welfare indicator in broilers (Ekstrand et al., 1997; Meluzzi et al., 2008). It is histologically validated and is easy to use for the routine assessment of broiler welfare in processing plants (Michela et al., 2012). A low prevalence and severity of FPD is highly desirable as far as the health of birds and product quality are concerned (Abd El-Wahab et al., 2012). Furthermore, the lesions can be a gateway for

276 bacteria that may spread hematogenously and impair product quality. Birds with severe
277 lesions may also show reduced weight gains, due to pain-induced decreases in feed intake
278 (Martland, 1985). FDP can be caused by several factors, the most important being the
279 condition of the litter in the broiler house (Bessei, 2006). Litter quality can be affected by diet
280 composition, as well as by the number and design of the drinkers in the pen (Lynn and Elson,
281 1990; Jones et al., 2005). The score obtained in the present study was zero for all of the birds,
282 thus showing that they were in a good welfare condition.
283 Intestinal morphology is the main indicator of gut health and functioning (Kristy et al., 2005).
284 In the present study, mealworm inclusion did not affect the morphology of the small intestine,
285 thus suggesting no influence on nutrient metabolization or performance. The greater
286 development of the duodenum, in relation to the other intestinal segments, is in agreement
287 with the results of Uni et al. (1999), Kondo (2003) and Murakami et al. (2007). In fact, the
288 duodenum is the intestinal tract with the fastest cell renewal, and is also the first segment of
289 the small intestine to receive physical, chemical and hormonal stimuli provoked by the
290 presence of the diet in the lumen (Macari, 1998).
291 In the present study, mealworm inclusion did not induce histological changes, thus suggesting
292 no negative influence on animal health. Glandular stomach alterations were considered
293 paraphysiological because, in the authors' opinion, they were probably related to the free-
294 range farming system. Outdoor access in fact entails less control of animal feeding, with the
295 possible ingestion of foreign bodies. However, the activation of the lymphoid tissue, in both
296 dietary treatments, represents the most interesting result. It has been reported that keeping
297 birds in free-range systems may favour the occurrence of gastrointestinal parasitic diseases,
298 with a high prevalence of coccidiosis and helminthiasis (Magwisha et al., 2002; Tomza-
299 Marciniak et al., 2014). Although all the birds were vaccinated against coccidiosis, the
300 immunological variation between strains of the same species might not have conferred total

protection (Chapman, 2014). Furthermore, the chronic flogosis observed in the intestinal segments could suggest previous parasitic infections, with subsequent activation of the lymphoid tissue. Tong et al. (2015) also evaluated the effects of outdoor access on the lymphoid organ index in a local chicken breed and found that the spleen was the only lymphoid organ that responded to outdoor access. The thymus, bursa of Fabricius and liver were not altered, but the possibility of an adaption of the birds to the environment, in terms of immune system modification, cannot be excluded.

In conclusion, the findings of this study suggest that the inclusion of yellow mealworm in free-range chicken diets does not affect the welfare, productive performance or morphological features of the birds. These results confirm previous data concerning the safety of TM meal use in poultry diets. A key point in future studies would be to establish the point of view of European consumers regarding the use of insects as feed for livestock. Legislative issues will also have to be addressed at a European level, in order to allow insect meal to be used as transformed animal protein to feed monogastric farm animals.

Acknowledgments

The authors are grateful to Mr. Alessandro Varesio and Mrs. Roberta Lacopo - “Fattoria La Fornace”, Montechiaro d'Asti (At - Italy) - for their technical support. The research was supported by the University of Torino (2014) and by the Regione Piemonte, Italy (PSR-PIAS no. 08000558869).

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493 **Figures captions**

494

495 **Figure 1.** Histological and morphometric evaluation of the gastrointestinal tract of the free-
496 range chickens. A) TM group. Morphometric measurements of the villus height (Vh) and the
497 crypt depth (Cd) in the jejunum segment. 2.5× H&E. B) TM group.. Severe and diffuse
498 mucosal lymphoplasmacytic and macrophagic flogosis with activation of the lymphoid tissue
499 (*) in the caecum. 5× H&E. C) C group. A normal glandular stomach. 5× H&E. D) C group.
500 Severe and diffuse mucosal lymphoplasmacytic flogosis with epithelial squamous metaplasia
501 (arrow) and activation of the lymphoid tissue (*) in the glandular stomach. 5× H&E.

502

503 **Figure 2.** Histological examination of the organs of the free-range chickens. A) TM group. A
504 normal follicle in the bursa of Fabricius. 10× H&E. B) TM group. Severe and diffuse
505 follicular depletion (*) in the bursa of Fabricius. 20× H&E. C) C group. A normal liver. 10×
506 H&E. D) C group. Mild and multifocal perivascular activation of the lymphoid tissue (arrow)
507 in the liver. 10× H&E.

508

509 **Tables**

510 **Table 1**. Ingredients and chemical composition of the experimental diets.

Ingredients (g/kg as fed)	Control diet	TM diet
Corn meal	720.0	720.0
Soybean meal	170.0	170.0
Tenebrio molitor meal	-	75.0
Gluten meal	75.0	-
Vitamin-mineral premix	35.0	35.0
Metabolizable energy (MJ/kg)	12.18	12.22
Analized composition		
Chemical composition (g/kg DM)		
Dry Matter	868	867
Crude Protein	169	168
Crude Fat	31	50
Crude Fiber	23	22
Indispensable aminoacids (g/kg DM)		
Arginine	9.3	10.0
Histidine	4.3	4.7
Isoleucine	7.1	6.9
Leucine	19.4	14.6
Lysine	7.0	9.0
Methionine	4.0	3.0
Phenylalanine	9.1	7.8
Threonine	7.0	7.0
Valine	8.1	8.2
Fatty acid composition (g/kg DM)		

C16:0	3.13	6.24
C18:0	0.47	1.06
C18:1 n9	4.00	10.05
C18:2 n6	10.42	20.41
C18:3 n3	0.52	0.73
Other fatty acids	0.35	1.12
Total SFA	3.66	7.75
Total MUFA	4.29	10.71
Total PUFA	10.94	21.15

*The mineral-vitamin premix (Trevit Volatili 3.5 - Trei - Rio Saliceto (RE) Italy) given values are supplied per kg: 650.000 IU of vitamin A; 65.000 IU of vitamin D3; 650 IU of vitamin E; 80 mg of vitamin K; 80 mg of vitamin B1; 150 mg of vitamin B2; 770 mg of vitamin B3; 80 mg of vitamin B6; 0.5 mg of vitamin B12; 240 mg of pantothenic acid; 4700 mg of betaine; 1750 mg of Iron (II) carbonate; 1835 mg of Magnesium oxide; 1612 mg of Zinc oxide; 178 mg of Copper (II) oxide; 18.3 mg of Potassium iodide; 6.6 mg of Sodium selenite; 4100 mg of DL-methionine; 5500 mg of L-lysine; 120 g Calcium carbonate; 450 g Calcium phosphate; 11.5 g of Sodium chloride. †SFA: saturated fatty acids; ‡MUFA: monounsaturated fatty acids; §PUFA: polyunsaturated fatty acids. ¶Other FAs (all less than 0.40 g/kg DM): C12:0, C14:0, C14:1 *cis*9, C16:1 *cis*9, C18:1 *cis*11, C20:0, C18:3 n6, C20:1 *cis*9, C20:1 *cis*11.

535 **Table 2.** Parameters evaluated for the histological semi-quantitative scoring system.

Organ	Alterations	Parameters evaluated
Spleen	White pulp hyperplasia	Number and dimension of follicles
	White pulp depletion	Number of apoptosis
		Cell types
Thymus	Cortical depletion	Cortico-medullar ratio
Bursa of Fabricius	Follicular depletion	Number of apoptosis
		Cell types
	Intrafollicular cysts	
Liver	Lymphoid tissue activation	Number of lymphoid aggregates
Glandular stomach	Lymphoplasmacytic flogosis	Distribution of inflammatory infiltrates
	Lymphoid tissue activation	Number and dimension of follicles
	Epithelial squamous metaplasia	
Intestine	Lymphoplasmacytic flogosis	Distribution of inflammatory infiltrates
	Lymphoid tissue activation	Number and dimension of follicles

536

537

538 **Table 3.** Growth and slaughtering performance of the free-range chickens (mean ± s.d.)

	Control diet	TM diet	*P*
Initial body weight (d 43) (g)	720.2 ± 26.81	712.3 ± 19.62	0.612
Final body weight (d 97) (g)	2131.0 ± 134.1	2162.5 ± 306.8	0.845
Average daily intake (g)	112.75 ± 9.9	111.6 ± 11.6	0.873
Feed conversion ratio	4.4 ± 0.7	4.4 ± 0.6	0.982
Mortality rate (%)	0	0	-
Footpad dermatitis score	0	0	-
Chilled carcass (g)	1459.3 ± 116.2	1544.8 ± 106.5	0.104
Breasts (g)	347.1 ± 41.6	370.8 ± 51.3	0.275
Thighs (g)	479.2 ± 45.3	502.9 ± 45.2	0.273
Thigh muscle (g)	349.3 ± 41.1	353.7 ± 27.7	0.793
Thigh bone (g)	83.8 ± 7.9	89.3 ± 9.2	0.186
Spleen (g)	4.1 ± 1.2	3.8 ± 1.1	0.615
Bursa of Fabricius (g)	4.3 ± 1.4	4.2 ± 2.1	0.873
Liver (g)	36.7 ± 4.0	39.4 ± 6.5	0.278
Gizzard (g)	66.7 ± 13.7	69.5 ± 9.8	0.603
Abdominal fat (g)	40.6 ± 35.4	45.1 ± 23.7	0.752

539

540

Table 4. Hematological and serum biochemical traits of the free-range chickens (mean ± s.d.).

	Control diet	TM diet	*P*
Erythrocytes (10^6 cell/µl)	2.40 ± 0.34	2.63 ± 0.38	0.641
Leukocytes (10^3 cell/µl)	9.42 ± 1.90	9.94 ± 1.74	0.659
H/L* ratio	0.55 ± 0.21	0.51 ± 0.22	0.798
Albumin (g/dl)	1.04 ± 0.24	1.31 ± 0.43	0.174
Total protein (g/dl)	3.98 ± 0.58	4.05 ± 0.79	0.293
AST † (UI/l)	190.49 ± 20.29	197.97 ± 15.16	0.649
ALT ‡ (UI/l)	14.49 ± 8.01	13.99 ± 5.64	0.411
Uric Acid (mg/dl)	4.99 ± 1.43	3.93 ± 1.34	0.894
Creatinine (mg/dl)	0.44 ± 0.05	0.46 ± 0.02	0.384
Triglycerides (mg/dl)	43.45 ± 17.98	47.81 ± 33.82	0.296
Cholesterol (mg/dl)	78.61 ± 17.09	78.90 ± 20.37	0.664
Glucose (mg/dl)	262.30 ± 39.45	243.60 ± 19.40	0.085
Phosphorus (mg/dl)	7.31 ± 4.99	6.45 ± 1.16	0.148
Magnesium (mEq/l)	0.90 ± 0.53	1.02 ± 0.53	0.979
Iron (µg/dl)	54.83 ± 37.03	61.54 ± 46.84	0.361

*H/L: heterophils/lymphocytes; †AST: aspartate-aminotransferase; ‡ALT: alanino-aminotransferase.

Table 5. Intestinal morphometric measurements of the free-range chickens.

	Control diet	TM diet	SEM	*P*
Duodenum				
1. Villus height (µm)	2.29[a]	2.49[a]	0.06	0.126
2. Crypt depth (µm)	0.18	0.21[d]	0.01	0.119
3. Villus height / crypt depth ratio	13.09[f]	12.17	0.64	0.491
Jejunum				
1. Villus height (µm)	1.94[b]	1.93[b]	0.08	0.945
2. Crypt depth (µm)	0.17	0.17[e]	0.01	0.813
3. Villus height / crypt depth ratio	11.29[fg]	11.14	0.44	0.875
Ileum				
1. Villus height (µm)	1.60[c]	1.72[b]	0.07	0.405
2. Crypt depth (µm)	0.17	0.17[e]	0.01	0.848
3. Villus height / crypt depth ratio	9.44[g]	10.27	0.31	0.180

Within each dietary treatment, different letters (a, b, c) in the same column mean significant differences ($P < 0.05$) among the intestinal segments (duodenum, jejunum and ileum) for villus height.

Within each dietary treatment, different letters (d, e) in the same column mean significant differences ($P < 0.05$) among the intestinal segments (duodenum, jejunum and ileum) for crypt depth.

Within each dietary treatment, different letters (f, g) in the same column mean significant differences ($P < 0.05$) among the intestinal segments (duodenum, jejunum and ileum) for villus height/crypt depth ratio.