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PhD THESIS
**INSECT MEAL UTILIZATION AS ALTERNATIVE PROTEIN
SOURCE IN POULTRY DIET: FOOD QUALITY AND SAFETY**

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*“Ho sceso, dandoti il braccio, almeno un milione di scale
e ora che non ci sei è il vuoto ad ogni gradino.
Anche così è stato breve il nostro lungo viaggio.
Il mio dura tuttora, né più mi occorrono
le coincidenze, le prenotazioni,
le trappole, gli scorni di chi crede
che la realtà sia quella che si vede.
Ho sceso milioni di scale dandoti il braccio
non già perché con quattr'occhi forse si vede di più.
Con te le ho scese perché sapevo che di noi due
le sole vere pupille, sebbene tanto offuscate,
erano le tue.”*

A te, nonna.

Abbreviations

AB: Alcian Blue
ADF: acid detergent fiber
ADG: average daily gain
ALT: alanine transaminase
AMEn: apparent metabolizable energy
AST: aspartate transaminase
C: control
Cd: crypt depth
CP: crude protein
DFI: daily feed intake
DM: dry matter
DU: duodenum
DWG: daily weight gain
EE: ether extract
FAs: fatty acids
FCR: feed conversion ratio
FDR: false discovery rate
GGT: gamma-glutamyltransferase
H/L: heterophils to lymphocytes ratio
HI: *Hermetia illucens*
HI10: 10% inclusion level of *Hermetia illucens*
HI15: 15% inclusion level of *Hermetia illucens*
HI5: 5% inclusion level of *Hermetia illucens*
HID: high iron diamine
IL: ileum
JE: jejunum
LW: live weight
MUFA: monounsaturated fatty acids
NDF: neuter detergent fiber
OTUs: operational taxonomic units
PAS: Periodic Acid-Schiff
PUFA: polyunsaturated fatty acids
SFA: saturated fatty acids
TM: *Tenebrio molitor*
TM10: 10% inclusion level of *Tenebrio molitor*
TM15: 15% inclusion level of *Tenebrio molitor*
TM5: 5% inclusion level of *Tenebrio molitor*
TM7.5: 7.5% inclusion level of *Tenebrio molitor*
Vh/Cd: villus height to crypt depth ratio
Vh: villus height

1 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). Subsequently, 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 nutritive needs of monogastric species, particularly fish and poultry, include protein sources that must have a high-protein content, an adequate amino acid profile, high digestibility, good palatability and no anti-nutritional factors (Barrows et al., 2008). The need of meeting up with the protein requirements is particularly important for poultry industry, which demands for animals capable of growing rapidly within a short period of time (Oyegoke et al., 2006). Indeed, protein sources represent the primary production costs in poultry diets (FAO, 2013).

Soybean meal is the most used vegetable protein source in diet formulations for broilers and laying hens, because of the high digestibility, high quality and quantity of protein and the best amino acid profile among the vegetable protein sources currently available (Veldkamp et al., 2012). However, due to its ever-increasing price, the sustainability of this production chain is becoming critical, in particular in some developing countries (FAO, 2013).

Fishmeal has also been widely used in poultry nutrition in the last decades, due to its excellent nutritive properties related to its very high nutrient density and digestibility. The protein in fishmeal has high biological value, because it is rich in essential amino acids, particularly lysine and sulphur-containing amino acids. In some developing countries, fishmeal is the most important conventional animal protein source, but its production availability and cost is a major issue (FAO, 2013).

Severe environmental concerns must also be considered in relation to the massive use of soybean and fishmeal. On one hand, the increased soy cultivation causes deforestation, high water consumption and utilization of chemicals. On the other hand, fishmeal quantitatively and qualitatively depends on catch, which is destined to decrease in the coming years due to the risk of marine resources depletion (Veldkamp et al., 2012; Sánchez-Muros et al., 2014). For all these reasons, the search for renewable and alternative sources of proteins in animal feeding is becoming fundamental.

2 INSECT MEAL AS ALTERNATIVE PROTEIN SOURCE OF FOOD FOR ANIMAL FEEDING

Insects are currently considered the novel protein source for animal feed (Premalatha et al., 2011; Veldkamp et al., 2012; Makkar et al., 2014; Sánchez-Muros et al., 2014). Indeed, insect meals exhibit a great potential for developing as a standard ingredient in animal feeding, because of their nutritive properties and rearing characteristics (Sánchez-Muros et al., 2014). All these aspects, which are going to be discussed in the present chapter, underline that it is technically feasible to produce insects on a large scale and to use them as alternative sustainable protein rich ingredient in animal diets.

2.1 Cultured species

The insect species currently cultured include butterflies and moths (O. *Lepidoptera*, more than 300 species have been raised), beetles (O. *Coleoptera*, more than 200 species), flies and mosquitoes (O. *Diptera*, approximately 200 species), bugs (O. *Heteroptera*, less than 100 species) and bees and wasps (O. *Hymenoptera*, less than 100 species). Crickets and grasshoppers (O. *Orthoptera*), lacewings (O. *Neuroptera*), cockroaches (O. *Blattodea*), termites (O. *Isoptera*) and fleas (O. *Siphonaptera*) are also reared (approximately 10-20 species) (Leppla, 2002). As reported by Makkar et al. (2014), the five major insect groups that can be potentially used as standard ingredients in animal feeding are represented by black soldier fly (*Hermetia illucens*), housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), locusts-grasshoppers-crickets and silkworm. Some of them are shown in Figure 1.

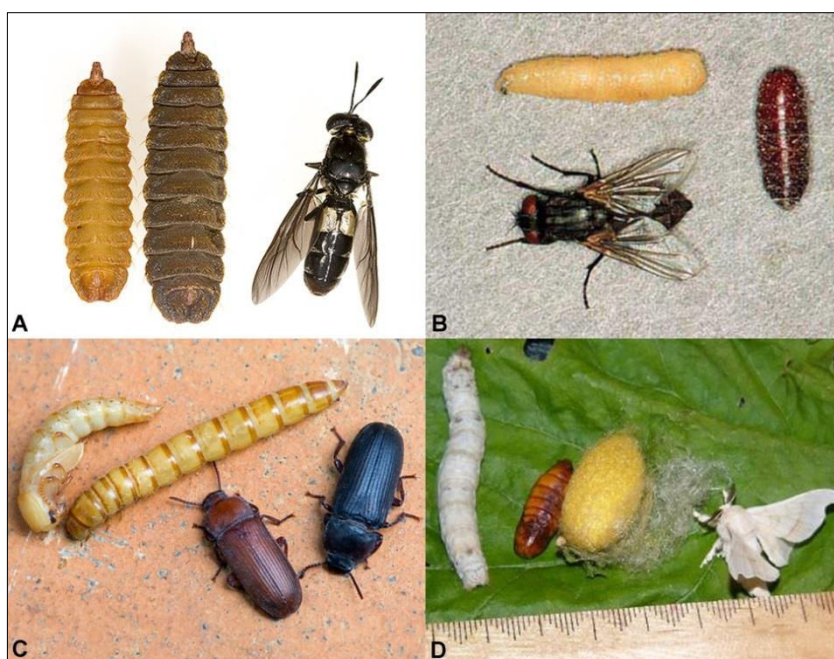


Figure 1. Larvae, pupae and adults of (A) *Hermetia illucens* (black soldier fly), (B) *Musca domestica* (housefly), (C) *Tenebrio molitor* (yellow mealworm) and (D) *Bombyx morii* (silkworm).

2.2 Mass-rearing

Mass production of insects is currently promising in an ecological perspective, since it can lead to a significant reduction of the 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). Firstly, insects are efficient food converters because they do not use energy to maintain a high body temperature (Nijdam et al., 2012). Furthermore, insects have different feeding habits and can be fed by-products (i.e., slaughterhouse, restaurant surpluses and cereal remnants), whose elimination has an economic and environmental cost. Certain insect species can also be easily grown on organic side streams, reducing environmental contamination and transforming waste into high-protein feed that can replace increasingly more expensive compound feed ingredients, such as fish meal. Another interesting aspect to consider is that culturing insects is usually performed in warehouses, with no need for large areas or much water, particularly when compared with crops. In addition, mass-rearing of insects contributes to the recycling of waste and determines relative low scores on carbon footprint (Blonk et al., 2008). However, although mass-rearing has been developed for some insect species, no systematic insect cultures have been developed, most likely because of a lack of demand. The culture of insects also shows some complications, because of their strict environmental (temperature and humidity), feeding and population requirements, particularly during reproduction (Leppla, 2002).

2.3 Nutritional values

Several studies have previously evaluated the nutritive value of insects, showing a variable proximal chemical composition. Indeed, the crude protein, lipids, nitrogen-free extract and ash contents can vary on varying species, phases of development (adult, larva or pupa) and rearing substrate (Sánchez-Muros et al., 2014). The proximate body composition of the previously mentioned most promising insect groups for animal feeding (black soldier fly, house fly, mealworm beetles, locusts–grasshoppers–crickets and silkworm) is summarized in Table 1.

Table 1. Chemical composition of the insect meals.

Insect meal	Crude protein (% in DM)	Crude fibre (% in DM)	Ether extract (% in DM)	Ash (% in DM)	Gross energy (MJ/kg DM)
Black soldier fly larvae (<i>Hermetia illucens</i>)	42.1 ± 1.0 (41.1, 43.6)	7.0	26.0 ± 8.3 (15.0, 34.8)	20.6 ± 6.0 (14.6, 28.4)	22.1
Housefly maggot (<i>Musca domestica</i>)	50.4 ± 5.3 (42.3, 60.4)	5.7 ± 2.4 (1.6, 8.6)	18.9 ± 5.6 (9.0, 26.0)	10.1 ± 3.3 (6.2, 17.3)	22.9 ± 1.4 (20.1, 24.4)
Housefly pupae (<i>Musca domestica</i>)	70.8 ± 5.3 (65.7, 67.2)	15.7	15.5 ± 1.0 (14.4, 16.1)	7.7 ± 2.1 (5.5, 9.8)	24.3
Yellow mealworm larvae (<i>Tenebrio molitor</i>)	52.8 ± 4.2 (47.2, 60.3)	NDF: 12.0 ± 3.5 (7.4, 15.0) ADF: 6.5 (6.4, 6.6)	36.1 ± 4.1 (31.1, 43.1)	3.1 ± 0.9 (1.0, 4.5)	26.8 ± 0.4 (26.4, 27.3)
Locust and / or grasshopper	57.3 ± 11.8 (29.2, 65.9)	8.5 ± 4.1 (2.4, 14.0)	8.5 ± 3.1 (4.2, 14.1)	6.6 ± 2.5 (4.3, 10.0)	21.8 ± 2.0 (19.5, 23.7)
House cricket (<i>Acheta domestica</i>)	63.3 ± 5.7 (55.0, 67.2)	NDF: 18.3 ± 2.9 (15.7, 22.1) ADF: 10.0 (9.6, 10.4)	17.3 ± 6.3 (9.8, 22.4)	5.6 ± 2.4 (3.6, 9.1)	N.A.
Field cricket (<i>Gryllus testaceus</i>)	58.1	N.A.	10.3	3.0	23.0
Mormon cricket (<i>Anabrus simplex</i>)	59.8 ± 4.1 (56.0, 65.4)	8.2 ± 1.1 (7.4, 9.8)	13.3 ± 5.0 (7.9, 19.9)	6.5 ± 1.9 (4.9, 9.0)	23.0

Values are expressed as mean ± standard deviation of data from previous studies. Values in parentheses are minimum and maximum values reported. DM, dry matter; NDF neutral detergent fibre; ADF acid detergent fibre. Source: Makkar et al. (2014).

2.3.1 Proteins

The percentage of protein is one of the most important criteria to be considered when choosing feed protein sources (Sánchez-Muros et al., 2014).

From a general point of view, most insect species have a high quality and quantity of protein (Ladrón de Guevara et al., 1995; Ramos-Elorduy et al., 1981, 1982, 1984 and 1997). As reported in Table 1, the crude protein (CP) contents are high (varying from 42 to 63%) and similar when compared with that of soybean meal (45-50%). However, CP proportions are slightly lower than that in fishmeal (60-80%) (Makkar et al., 2014). The defatting process may further influence the insects CP content, with the obtaining of higher values than soybean meal and fishmeal. Indeed, black soldier fly larvae, housefly maggot, mealworm and silkworm meals have been reported to contain as high as 36% oil, which can be isolated and used for the preparation of biodiesel. Subsequently, the rest of the defatted meal, being rich in CP, could be employed as an invaluable protein-rich resource in the feed industry (Makkar et al., 2014).

2.3.2 Amino acids

Rather than a true protein requirement, animals have an amino acid requirement, which proteins are composed of (Teles et al., 2011). Therefore, one criterion to define protein quality is the amino acid composition, particularly the balance between essential and nonessential amino acids (Conconi et al., 1984).

Insects are generally rich in essential amino acids (Makkar et al., 2014; Sánchez-Muros et al., 2014). This represents an advantage in comparison to the vegetable protein sources, which are usually deficient lysine, methionine and leucine (the more frequent limiting amino acids) (Hall, 1992). On the contrary, despite housefly maggot, housefly pupae and silkworm pupae meals containing higher levels of methionine in comparison with that of fishmeal, none of them raise its amino acid composition (Sánchez-Muros et al., 2014).

The detailed amino acid compositions of the previously mentioned most promising insect groups for animal feeding (black soldier fly, house fly, mealworm beetles, locusts–grasshoppers–crickets and silkworm) are reported in Table 2. Methionine levels in all insect meals are higher than in soybean meal, while the levels of sulphur-containing amino acids (methionine and cystine) are lower in black soldier fly larvae, mealworm, house cricket and mormon cricket meals compared with that of soybean meal. Lysine is lower in mealworm, locust, house cricket and mormon cricket meals than in soybean meal. Lysine levels are adequate in black soldier fly larvae, housefly maggot and silkworm pupae meals (Makkar et al., 2014), while high levels of leucine are detected in housefly maggot, housefly pupae and silkworm pupae meals (Sánchez-Muros et al., 2014). Overall, most essential amino acid levels in silkworm pupae and black soldier fly larvae meals are higher when compared with that of

soybean meal or the FAO Reference Protein. Arginine is also considered an essential amino acid for laying hens and the level of this amino acid in all insect meals has been reported to be lower than in soybean meal, suggesting the importance of its addition in the diets of laying hens containing insects (Makkar et al., 2014).

Table 2. Amino acid composition of the insect meals.

Insect meal	Essential Amino Acids (g/16g nitrogen)										Non Essential Amino Acids (g/16g nitrogen)							
	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Asp	Cys	Glu	Gly	Pro	Ser	Tyr	Ala
Black soldier fly larvae (<i>Hermetia illucens</i>)	5.6 ± 0.3 (5.3, 6.1)	3.0 ± 1.0 (2.3, 4.5)	5.1 ± 0.5 (4.7, 5.6)	7.9 ± 0.6 (7.1, 8.4)	6.6 ± 0.9 (6.0, 8.0)	2.1 ± 0.3 (1.7, 2.4)	5.2 ± 0.4 (4.6, 5.6)	3.7 ± 1.7 (1.3, 4.8)	0.5	8.2 ± 1.4 (6.4, 9.1)	11.0 ± 1.8 (8.5, 12.5)	0.1	10.9 ± 2.4 (8.7, 13.5)	N.A.	6.6 (5.5, 7.7)	3.1 ± 1.9 (0.3, 4.2)	6.9 ± 0.7 (6.0, 7.7)	7.7 ± 0.8 (6.9, 8.8)
Housefly maggot (<i>Musca domestica</i>)	4.6 ± 0.7 (3.7, 5.8)	2.4 ± 0.8 (1.0, 3.6)	3.2 ± 0.5 (2.3, 3.7)	5.4 ± 0.6 (4.5, 6.4)	6.1 ± 0.9 (5.0, 8.2)	2.2 ± 0.8 (1.3, 3.7)	4.6 ± 0.8 (3.7, 5.9)	3.5 ± 0.7 (2.0, 4.1)	1.5 (1.4, 1.5)	4.0 ± 1.1 (1.3, 4.9)	7.5 ± 1.5 (4.5, 8.5)	0.7 ± 0.2 (0.5, 1.0)	11.7 ± 1.8 (8.9, 15.3)	4.2 ± 0.4 (3.7, 5.1)	3.3 ± 0.7 (2.5, 4.7)	3.6 ± 0.5 (2.6, 3.9)	4.7 ± 1.4 (2.9, 7.1)	5.8 ± 1.0 (4.4, 7.6)
Housefly pupae (<i>Musca domestica</i>)	4.9 ± 0.9 (4.2, 5.9)	2.0 ± 0.6 (1.5, 2.6)	3.4 ± 0.2 (3.2, 3.5)	5.2 ± 0.3 (4.9, 5.4)	5.5 ± 0.9 (4.8, 6.5)	2.0 ± 0.6 (1.5, 2.6)	4.2 ± 0.5 (3.7, 4.7)	3.2 ± 0.2 (3.0, 3.4)	N.A.	4.2 ± 0.7 (3.4, 4.6)	7.9 ± 1.2 (6.5, 8.7)	0.4	10.2 ± 2.1 (7.8, 12.0)	4.1 ± 0.2 (3.9, 4.3)	3.4 (3.1, 3.7)	3.1 ± 0.4 (2.7, 3.4)	4.9 ± 0.4 (4.6, 5.3)	4.2 ± 0.2 (4.1, 4.4)
Yellow mealworm larvae (<i>Tenebrio molitor</i>)	4.8 ± 1.0 (3.8, 5.6)	3.4 ± 0.2 (3.2, 3.6)	4.6 ± 0.5 (4.1, 5.0)	8.6 ± 1.8 (7.4, 10.6)	5.4 ± 0.8 (4.6, 6.1)	1.5 ± 0.4 (1.3, 2.0)	4.0 ± 0.4 (3.5, 4.3)	4.0 ± 0.5 (3.5, 4.4)	0.6 ± 0.5 (0.0, 0.9)	6.0 ± 0.6 (5.5, 6.6)	7.5 ± 1.7 (5.6, 8.8)	0.8 ± 0.0 (0.8, 0.9)	11.3 ± 1.1 (10.2, 12.4)	4.9 ± 0.9 (3.9, 5.6)	6.8 ± 0.2 (6.6, 7.0)	7.0 ± 3.5 (4.9, 11.1)	7.4 ± 0.3 (7.1, 7.8)	7.3 ± 1.0 (6.2, 8.2)
Locust and / or grasshopper	5.6 (3.7, 7.4)	3.0 (1.9, 4.2)	4.0 (3.9, 4.1)	5.8 (5.6, 5.9)	4.7 (3.4, 5.9)	2.3 (2.3, 2.3)	3.4 (2.3, 4.5)	3.5 (2.9, 4.0)	0.8	4.0 (3.8, 4.3)	9.4	1.1 (0.5, 1.7)	15.4	4.8 (4.7, 4.8)	2.9 (2.1, 3.8)	5.0	3.3 (3.1, 3.6)	4.6 (4.1, 5.1)
House cricket (<i>Acheta domestica</i>)	6.1 (6.1, 6.1)	2.3 (2.2, 2.3)	4.4 (4.3, 4.6)	9.8 (9.5, 10.0)	5.4 (5.4, 5.4)	1.4 (1.3, 1.5)	3.0 (2.8, 3.2)	3.6 (3.6, 3.6)	0.6 (0.5, 0.6)	5.1 (4.9, 5.2)	7.7 (7.1, 8.4)	0.8 (0.8, 0.8)	10.4 (10.4, 10.5)	5.2 (5.1, 5.3)	5.6 (5.5, 5.6)	4.6 (4.2, 5.0)	5.2 (4.9, 5.5)	8.8 (8.8, 8.9)
Field cricket (<i>Gryllus testaceus</i>)	3.7	1.9	3.1	5.5	4.8	1.9	2.9	2.8	N.A.	4.4	6.3	1.0	9.1	3.6	4.5	3.7	3.9	5.6
Mormon cricket (<i>Anabrus simplex</i>)	5.3 (4.5, 6.0)	3.0 (2.6, 3.3)	4.8 (4.2, 5.3)	8.0 (7.3, 8.6)	5.9 (5.6, 6.2)	1.4 (1.3, 1.5)	2.5 (2.2, 2.8)	4.2 (3.5, 4.8)	0.6 (0.5, 0.6)	6.0 ± 0.6 (5.5, 6.6)	8.8	0.1	11.7	5.9	6.2	4.9	5.2 (4.1, 6.2)	9.5

Values are expressed as mean ± standard deviation of data from previous studies. Values in parentheses are minimum and maximum values reported. Arg, arginine; His, histidine; Ile, isoleucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Thr, threonine; Trp, tryptophan; Val, valine; Asp, aspartic acid; Cys, cystine; Glu, glutamic acid; Gly, glycine; Pro, proline; Ser, serine; Tyr, tyrosine; Ala, alanine. Source: Makkar et al. (2014).

2.3.3 Lipids and fatty acids

The final composition of animal lipids reflects the fatty acid composition of the diet, which affects the quality and healthy properties of the final product, such as muscle (Sánchez-Muros et al., 2014). The detailed fatty acid compositions of the previously mentioned most promising insect groups for animal feeding (black soldier fly, house fly, mealworm beetles, locusts–grasshoppers–crickets and silkworm) are summarized in Table 3. Unsaturated fatty acids (monosaturated and polyunsaturated) concentrations are high in mealworm, house cricket and housefly maggot meals (60–70%), but lowest in black soldier fly larvae meal (19–37%), thus suggesting the presence of higher levels of saturated fatty acids in the latter. This is indicative of a undesirable lipid profile, since dietary deficiencies of polyunsaturated fatty acids such as linoleic (18:2), linolenic (18:3) and arachidonic (20:4) may lead to a number of adverse effects (i.e., growth retardation, dermal symptoms, malabsorption and catabolic diseases) (Simopoulos, 1999). However, black soldier fly can be subjected to fatty acid composition manipulation by changing the composition of the substrate during the culture (St-Hilaire et al., 2007).

Table 3. Fatty acid composition of the insect meals.

Insect meal	Saturated fatty acids (%)				Monosaturated fatty acids (%)		Polyunsaturated fatty acids (%)			
	Lauric (12:0)	Myristic (14:0)	Palmitic (16:0)	Stearic (18:0)	Palmitoleic (16:1 n -7)	Oleic (18:1 n -9)	Linoleic (18:2 n -6)	Linolenic (18:3 n -3)	EPA (20:5 n -3)	DHA (22:6 n -3)
Black soldier fly larvae (<i>Hermetia illucens</i>)	21.4 [49.3] (42.6)	2.9 [6.8] (6.9)	16.1 [10.5] (11.1)	5.7 [2.78] (1.3)	[3.5]	32.1 [11.8] (12.3)	4.5 [3.7] (3.6)	0.19 [0.08] (0.74)	0.03 [0] (1.66)	0.006 [0] (0.59)
Housefly maggot (<i>Musca domestica</i>)	N.A.	5.5	31.1	3.4	13.4	24.8	19.8	2.0	N.A.	N.A.
Yellow mealworm larvae (<i>Tenebrio molitor</i>)	0.5	4.0	21.1	2.7	4.0	37.7	27.4	1.2	N.A.	N.A.
House cricket (<i>Acheta domestica</i>)	N.A.	0.7	23.4	9.8	1.3	23.8	38.0	1.2	N.A.	N.A.

Values using cow manure as substrate. Round parentheses are the values obtained on using 50% of cow manure and 50% of fish offal as substrate. Square parentheses are values obtained on swine manure as substrate. Source: Makkar et al., 2014.

2.3.4 Digestibility

Along with CP content and amino acid composition, another important characteristic of protein quality is the digestibility of the source (Sánchez-Muros et al., 2014).

The digestibility of insect proteins and their utilization *in vivo* have shown promising results. Apparent faecal CP digestibility of black soldier fly larvae meal and soybean meal in male growing pigs has been reported to be similar (76 and 77%, respectively) (Newton et al., 1977). Good apparent digestibility coefficient of the total tract of CP of full-fat (51%), partially defatted (62%) and highly defatted (62%) black soldier fly meal has recently been observed in broiler chickens (De Marco et al., 2015; Schiavone et al., 2017a). The same authors also reported an apparent ileal digestibility coefficient of amino acid ranging from 42 to 89% for the full-fat meal (De Marco et al. 2015), from 44 to 92% for the partially defatted meal and from 45 to 99% for the highly defatted meal (Schiavone et al., 2017a). About housefly meal utilization in broilers, Pretorius (2011) observed apparent faecal CP digestibility of 69%, while Hwangbo et al. (2009) reported a higher value of 98.5%. The former study also observed amino acid digestibility of > 90%, while the CP digestibility was much lower, which might be attributed to indigestibility of chitin-N and/or acid detergent fiber (ADF) bound-N. De Marco et al. (2015) have also recently described high apparent digestibility coefficient of the total tract of CP (60%) and high apparent ileal digestibility coefficient of amino acid (80-93%) of full-fat yellow mealworm meal in broiler chickens.

2.3.5 Indigestible components: chitin

Chitin is a polysaccharide that is present exclusively in the exoskeleton of arthropods (van Huis, 2013; Sánchez-Muros et al., 2014). It is a non-toxic; biodegradable linear polymer composed mainly of β -(1 $\rightarrow$ 4)-linked 2-acetamido-2-deoxy- β -D-glucopyranose units and partially of β -(1 $\rightarrow$ 4)-linked 2-amino-2-deoxy- β -D-glucopyranose (van Huis, 2013).

As a crude fibre (Lindsay et al., 1984), chitin is difficult to digest by domestic poultry (Ravindran and Blair, 1993). Indeed, it has been reported to negatively influence the nutrient digestibility of crude protein (Razdan and Pettersson, 1994; Hossain and Blair, 2007) and organic matter (Razdan and Pettersson, 1994). However, recent studies observed that chitin has complex and size-dependent effects on innate and adaptive immune responses (Lee et al., 2008). Indeed, chitin has immune-system stimulating properties and its ingestion could improve the immune status of the animals. As a consequence, insect meal utilization in chickens could potentially contribute to a reduction of the use of antibiotics in poultry industry and a diminishment of human infection by drug-resistant bacterial strains (van Huis et al., 2013).

3 INSECT MEAL UTILIZATION IN POULTRY FEED

The feeding studies conducted so far have shown the feasibility of insect-derived feeds as a useful alternative to soybean meal and fishmeal in poultry nutrition (van Huis, 2013; Makkar et al., 2014; Sánchez-Muros et al., 2014). These studies, which are going to be summarized in the current chapter, have demonstrated that the palatability of insect meals is good and these alternate feed resources can potentially replace soybean and fishmeal in the diets of chickens (Makkar et al., 2014).

3.1 Black soldier fly larvae (*Hermetia illucens*)

Previous studies conducted on including black soldier fly larvae meal in poultry diets suggest that it could replace soybean meal, even if its inclusion level needs to be optimized (Makkar et al., 2014). Chicks fed a diet containing dried black soldier fly larvae (as a substitute for soybean meal) gained weight at a rate 96% (even if non-significant) of that of birds fed the control diet, but they only consumed 93% (significant) as much feed (Hale, 1973). This suggests a higher feed conversion efficiency of diet containing the black soldier fly larvae meal (Makkar et al., 2014). Cullere et al. (2016) recently studied the influence of the inclusion (from 10 to 15%) of *Hermetia illucens* meal in broiler quails diet, finding no differences for growth performance and carcass traits. The same authors also performed a feed-choice test and observed that the birds tended to prefer the diets including black soldier fly larvae meal. Laying hens fed diets in which the soybean meal was completely replaced by *Hermetia illucens* larvae meal showed a more favorable feed conversion ratio than birds fed the control diet, but lower lay percentage, feed intake, average egg weight and egg mass (Marono et al., 2017). Loponte et al. (2017) also investigated the effects of the replacement (from 25 to 50%) of soybean meal with black soldier fly larvae meal in Barbary partridges diet, observing higher live and carcass weights in the birds fed with *Hermetia illucens* meal.

3.2 Housefly maggot (*Musca domestica*)

Previous trials about maggot meal inclusion in broiler diets as a replacement for conventional protein sources indicate that partial or even total replacement of fishmeal is possible, even if the optimal inclusion rate seems to be lower than 10% in the diet (Makkar et al., 2014).

Adeniji (2007) and Hwangbo et al. (2009) studied the effects of housefly maggots as feed supplement in the diet of broiler chickens: the first found no differences for growth performance with inclusion levels ranging from 5.5 to 22%, while the latter (inclusion rate: 5-20%) observed a linear increase in the live weight gain and improved eviscerated carcass weights. Similarly to Hwangbo et al. (2009), Téguia et al. (2002) determined that the total weight gain in the broilers fed with fishmeal was lower

than that of birds fed diets containing *Musca domestica* maggots. Khan et al. (2017) also observed that broiler chickens fed with housefly maggot meal showed higher body weight and lower feed conversion ratio in comparison with those fed with soybean meal. However, the majority of the studies did not find any significant differences in weight increase between broilers fed with housefly maggot meal or high quality fishmeal (Ocio and Vinaras, 1979; Awoniyi et al., 2003; Djordjevic et al., 2008). Live maggots have also been reported to be a valuable supplement to the diet of rural chickens. Indeed, the supplementation of 30–50 g/day/bird of live maggots to scavenging backyard chickens in Ghana resulted in higher growth rate (until the 5th month), clutch size, egg weight, number of eggs hatched and chick weight (Dankwa et al., 2002). A similar preliminary test in Togo was also promising, as the chickens seemed particularly fond of moving larvae (Ekoue and Hadzi, 2000). In a 7-month layer feeding trial in laying hens, maggot meal increased egg yield and hatchability when included in the diet as a replacement of meat and bone meal (Ernst et al., 1984). In 50-week laying hens, maggot meal has been reported to replace 50% of fishmeal protein (5% in diet) without any adverse effects on egg production and shell strength. However, 100% replacement was deleterious to hen-egg production (Agunbiade et al., 2007).

3.3 Yellow mealworm (*Tenebrio molitor*)

The information so far available suggests that yellow mealworm could be a valuable replacer of soybean meal and fishmeal in poultry diets (Makkar et al., 2014).

Ramos-Elorduy et al. (2002) showed no effects for the growth performance in fast-growing chickens fed sorghum-soybean meal-based diets in which the full-fat *Tenebrio molitor* meal inclusion level ranged from 5 to 10% in partial substitution of soybean meal and vegetable oil. Differently, Ballitoc and Sun (2013) and Bovera et al. (2015 and 2016) observed improved growth performance in fast-growing chickens fed diets with low (from 0.5 to 10%) or high (29.6%) yellow mealworm inclusion levels, respectively. Similar findings were reported by Loponte et al. (2017) in Barbary partridges fed diets in which the *Tenebrio molitor* meal inclusion level ranged from 25 to 50%. Furthermore, Kahn et al. (2017) observed higher body weight and lower feed conversion ratio in broiler chickens fed with yellow mealworm than those fed with soybean meal, with reduced feed intake also found in yellow mealworm group when compared with control, housefly maggot meal and silkworm meal groups. Recently, Islam and Yang (2017) also found a positive effect of a mealworm-based probiotic (*Tenebrio molitor* and *Zophobas morio* with *Lactobacillus plantarum* KCTC 3099 and *Saccharomyces cerevisiae* KCTC 7928) on broiler growth performance. Improved slaughter, dressed carcass and eviscerated weights (Ballitoc and Sun, 2013), carcass weights (Loponte et al., 2017) or unaffected carcass traits (Bovera et al., 2016) were also observed.

3.4 Locusts, grasshoppers and crickets

Previous studies have tried to replace a part of fishmeal with locust and grasshopper meal and found that such partial substitution is generally suitable (Makkar et al., 2014).

Broiler chickens (1–28 days) fed desert locust meal (*Schistocerca gregaria*) as a replacement of 50% fishmeal protein (inclusion level of 1.7% in the diet) showed higher body weight gain, feed intake and feed conversion ratio (Adeyemo et al., 2008). Grasshopper meal from *Acrida cinerea* has been reported to replace 20% and 40% fishmeal in broiler diets with similar growth rate and feed consumption as the control diet (Liu and Lian, 2003). Similarly, Wang et al. (2007) observed that the inclusion of 15% *Acridia cinerea* meal in broiler chicken diets did not affect growth performance. Differently, grasshopper meal from unspecified species at the inclusion levels of 2.5–7.5% in broiler diets (1–49 days) has been reported to depress weight gain and feed efficiency, even if it increased the protein content of the carcass (Ojewola et al., 2003). In a more recent study, Ojewola et al. (2005) observed that grasshopper meal included at 2.5% in the diet was a suitable and cheaper substitute for imported fishmeal (100% replacement level), despite the overall diet containing slightly less protein (22.2% vs 22.8%).

Several studies have also investigated the effects of utilization of Mormon cricket (*Anabrus simplex*) meal in broiler diets. Broiler chickens (1–21 days) fed a diet of 62% maize grain and 30% cricket meal showed better growth performance than broilers fed a control diet based on maize, fishmeal and meat and bone meal. Palatability was also not affected (DeFoliart et al., 1982). Finke et al (1985) also evaluated the effects of maize-cricket diets in which soybean meal was totally replaced with cricket meal (inclusion levels of 28, 22 and 18%) in broilers up to 8 weeks, finding no significant differences in weight gain and feed conversion ratio between birds fed the maize-cricket diet and those fed a maize-soybean meal diet. A similar experiment with a diet based on maize and 25% house crickets (*Acheta domestica*) fed to broilers for 2 weeks showed that cricket-based diets led to higher feed conversion ratios than the control (maize-soybean meal based) diet (Nakagaki et al., 1987). Wang et al. (2005) also reported that field cricket (*Gryllus testaceus*) could be included up to 15% in the diet (as a partial replacement of soybean meal) of 8–20 day broilers without any adverse effects on weight gain, feed intake or feed conversion ratio.

3.5 Silkworm pupae

Limited studies evaluating the effects of silkworm pupae utilization in poultry feed are currently available (Makkar et al., 2014).

Ijaiya and Eko (2009) investigated the influence of replacing dietary fishmeal with silkworm meal from *Anaphe infracta* (inclusion rate: 2.2-9.3%) on growth performance of broiler chickens, observing no significant differences. Differently, Khatun et al. (2003) observed a linear increase of growth rate, feed

conversion ratio, livability and meat yield with increasing levels (2, 4 and 6%) of silkworm pupae as a replacement of dietary fishmeal in broilers. Khan et al. (2017) also reported that broiler chickens fed with silkworm meal showed higher body weight and lower feed conversion ratio in comparison with those fed with soybean meal. Silkworm meal has also been reported to be well accepted by laying hens (Saikia et al., 1971; Joshi et al., 1979 and 1980). On the contrary, Mahanta et al. (2004) observed that silkworm pupae meal was detrimental to the breeding performance of breeder males.

4 DIETARY MODIFICATIONS AND GUT HEALTH IN POULTRY

Insect meal utilization can be contextualized within the wider topic of dietary modifications, which has a key role in poultry industry. Generally, the parallel evaluation of the nutrient digestibility, growth performance and carcass traits represents the standard nutritional and zootechnical approach for the assessment of the impact of a new diet in poultry. However, a better understanding of the effects of dietary manipulations can be provided by the *post mortem* evaluation of the intestine. In particular, the concept of “gut health” is becoming progressively more important, not only in chickens, but also in other production animals. After a brief description of the anatomy and histology of the chicken intestine, the present chapter will focus its attention on gut health definition and markers in poultry.

4.1 Anatomy and histology of chicken intestine

The chicken intestine (Figure 2) is generally shorter in relative terms than in domestic mammals. The small intestine is comprised of the duodenum, jejunum and ileum, with poorly differentiated boundaries being identified between these intestinal segments. Considering the lack of a morphological distinction between the jejunum and the ileum, these segments are sometimes referred as the “jejunoileum”. The large, or terminal, intestine includes the caeca and the rectum (König et al., 2016).

The duodenum begins at the *ostium ventriculo-pyloricum* of the ventriculus and forms a U-shaped *ansa duodeni* consisting of a descending portion (the *pars descendens*) and an ascending component (the *pars ascendens*). Lying between the two portions is the pancreas. Differently from mammals, the ascending duodenum typically accommodates the openings of three pancreatic ducts and two bile ducts, the *ductus hepatoentericus* and the *ductus cysticoentericus*. These ducts open at or near the *papilla duodeni* (König et al., 2016).

Located just cranial to the cranial mesenteric artery, the *flexura duodenojejunalis* forms the junction between the duodenum and the jejunum. The jejunum and ileum are arranged in loops occupying the right caudal quadrant of the body cavity. The Meckel’s diverticulum (*diverticulum vitellinum*), an embryonic remnant of the yolk stalk, is located on the *ansa axialis* (an intestinal loop in the middle of the jejunoileum), opposite the longest middle branch of the cranial mesenteric artery. This rudimentary structure is often considered to represent the boundary between the jejunum and ileum, although there are no discernible morphological differences upon which to base such a distinction (König et al., 2016).

Differently from mammals, domestic poultry have two large-sized caeca. They begin at the transition between the ileum and the rectum and are connected to the terminal ileum by a well-defined ileocaecal ligament. Each caecum communicates with the rectum via an *ostium caeca*. A muscular sphincter is present at the base (*basis caeca*) of each caecum. The caeca are particularly well

developed in chickens. At its base, each caecum is richly endowed with lymphatic tissue, sometimes referred to as the caecal tonsils. The caecal body (*corpus caeca*) is thin-walled and often ampulliform, while the apex (*apex caeca*) can be pointed or vesicular. In herbivorous and frugivorous species, the caeca are the site of breakdown of cellulose. The rectum is the final, straight segment of the intestine that passes to the cloaca. Until recently, it was referred to as the colon (König et al., 2016).

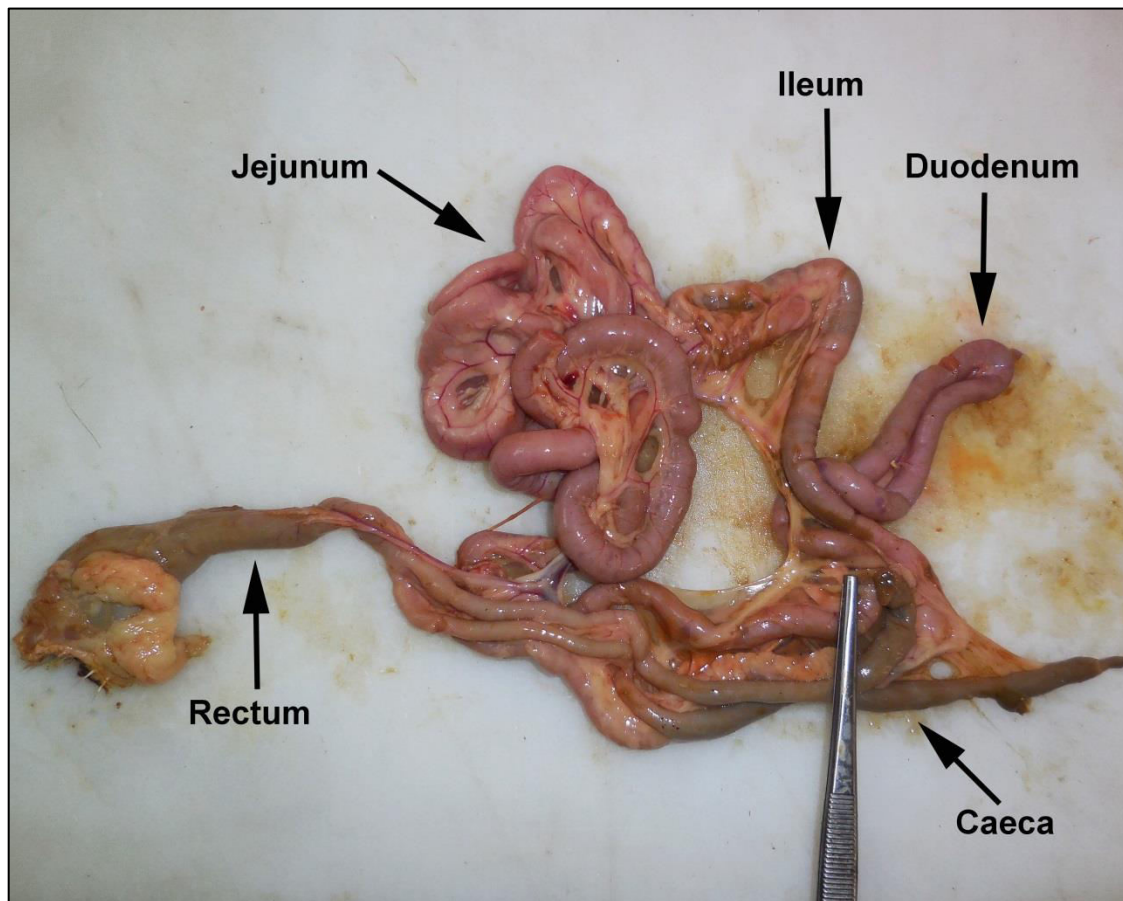


Figure 2. Gross picture of chicken intestine after slaughter. All the different segments can be easily appreciated.

The intestinal wall (Figure 3) is histologically composed of four coats, each of them is called either a tunica or a tela: tunica mucosa, submucosa, muscularis and serosa (Frappier, 2006).

The tunica mucosa includes the lining epithelium, a lamina propria with glands, and a lamina muscularis. The villi are mucosal projections and represent the most characteristic feature of the small intestine. The intestinal glands (crypts of Lieberkühn) open between the bases of the villi and penetrate the mucosa as far as the lamina muscularis. The lumen of the small intestine is covered by a simple columnar epithelium containing numerous goblet cells interspersed among columnar absorptive cells. The crypts of Lieberkühn are simple tubular glands that are lined by a variety of cell types. The principal cell type is represented by the undifferentiated columnar cells, which multiply, differentiate and migrate into the villus, giving rise to the columnar absorptive cells and the goblet

cells. The lamina propria, which forms the cores of the villi and surrounds the intestinal glands, is composed of loose connective tissue with a prominent reticular fiber framework. Within the lamina propria, blood and lymph vessels, leukocytes, fibrocytes, smooth muscle cells, plasma cells and mast cells are generally observed. The lamina muscularis is composed of inner circular and outer longitudinal layers of smooth muscle that tend to be thin and incomplete. The large intestine is characterized by the absence of villi and the presence of longer, less-coiled, simple tubular intestinal glands with many goblet cells (Frappier, 2006).

The submucosa is a layer of dense connective tissue (Frappier, 2006). Diffuse lymphatic tissue (the gut-associated lymphatic tissue or GALT) is generally present in the lamina propria and the submucosa (Aughey and Frey, 2001).

The tunica muscularis is composed of inner circular and outer longitudinal smooth muscle layers (Frappier, 2006), with a third layer of circular smooth muscle being sometimes detected in the muscularis externa (Aughey and Frey, 2001). The connective tissue between the muscle layers contains the myenteric plexus (Frappier, 2006).

The tunica serosa covers the entire intestine and consists of a layer of loose connective tissue lined by mesothelium (Frappier, 2006).

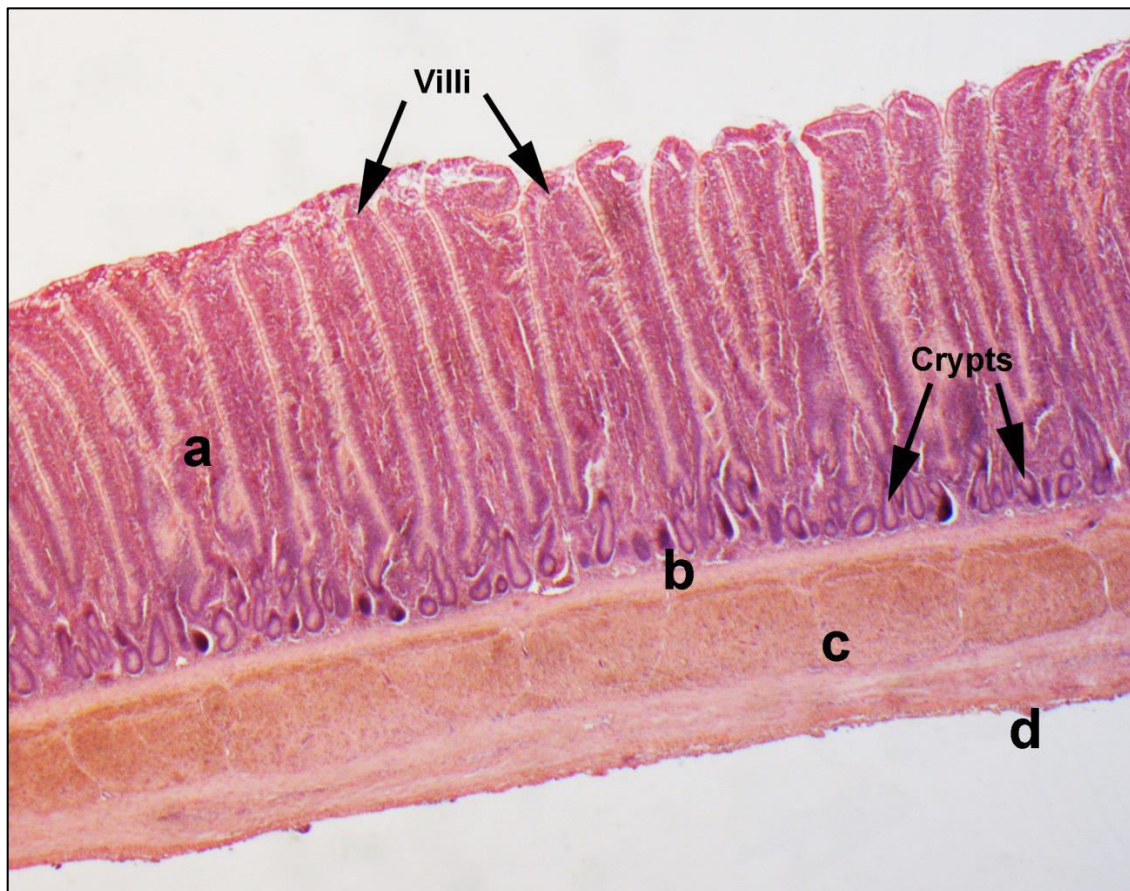


Figure 3. Histological picture of chicken ileum. The four tunics can be observed: (a) tunica mucosa, (b) tunica submucosa, (c) tunica muscularis and (d) tunica serosa. Haematoxylin & Eosin stain, 2.5× magnification.

4.2 Gut health: definition and markers

Gut health has been a focus of major research efforts in production animals in the last decades, since it can be considered a synonymous to animal health and it is of vital importance to animal performance (Kogut and Arsenault, 2016). The latter aspect has a key role in poultry industry, which requires animals capable of growing rapidly within a short period of time (Oyegoke et al., 2006). Gut health depends on the maintenance of the delicate balance between the host, intestinal microbiota, intestinal barrier and dietary compounds (Bailey, 2013). In particular, gut microbiota benefits the host by providing nutrients from otherwise poorly utilized dietary substrates and modulating the development and function of the digestive and immune system (Pan and Yu, 2014). Firstly, gut microbiota alterations can affect intestinal morphology through modifications of villus height and crypt depth (Forder et al., 2007), which are considered the main indicators of gut development, health and functionality (Wang and Peng, 2008). There is also evidence that gut microbiota may modulate synthesis and composition of mucins (Robertson and Wright, 1997; Mack et al., 1999; Forder et al., 2007), which constitute a digestion- and absorption-assisting medium and represent the first line of defense for intestinal epithelium against foreign bacteria and other pathogens (Forstner and Forstner, 1994). Several feed substances have been reported to widely affect gut health in poultry, either by directly modifying intestinal morphology (Laudadio et al., 2012; Qaisrani et al., 2014) and mucin composition (Tsirtsikos et al., 2012a and 2012b), or indirectly by modulating intestinal microbiota (Pan and Yu, 2014; Ranjitkar et al., 2016). Therefore, the simultaneous evaluation of these three aspects together seems to be fundamental in chickens.

4.2.1 Gut mucosal morphology

Features of the gastrointestinal tract have been reported to influence the efficiency of utilization of dietary protein (Swatson et al., 2002), which is considered a crucial regulator of poultry growth and reproductive performance (Laudadio et al., 2012). In particular, the small intestinal morphology (especially regarding crypts and villi of the absorptive epithelium) is considered the main indicator of intestinal development, health and functionality, such influencing nutrient digestion and absorption (Wang and Peng, 2008). Particular attention is given to crypt and villus since the former is the region in which new intestinal cells are formed and the latter has a fundamental role in the digestion and absorption of nutrients (Swatson et al., 2002). Morphometric measurements of villus height and crypt depth are generally used to assess intestinal development (Franco et al., 2006), since they represent useful indicators of gut proliferative and absorptive compartments (Lenhardt and Mozes, 2003). The villus height to crypt depth ratio is also evaluated, because it gives an indication of the likely maturity and functional capacity of the enterocytes (Hampson, 1986). The ideal gut morphological asset appears to be characterized by long villi and shallow crypts. Indeed, longer villi are generally

associated with increased total luminal absorptive area and subsequent satisfactory digestive enzyme action and higher transport of nutrients (Laudadio et al., 2012). In parallel, shallower crypts reflect the prolonged survival of villi without the need for renewal (Oliveira et al., 2008), with reduced energy expenditure for this process and consequent enabled growth of other tissues (Miles et al., 2006). Differently, lower villus heights and greater crypt depths lead to poor digestion and less absorption of nutrients and, as a consequence, poor performance of the animals (Qaisrani et al., 2014). Dietary modifications have been reported to widely affect the intestinal development and the mucosal architecture of the gastrointestinal tract of broiler chickens fed with different cereal grains and microbial enzyme supplementation (Shakouri et al., 2009), polyphenol-rich grape products (Viveros et al., 2011), different protein levels (Laudadio et al., 2012), crude glycerol (Ozdogan et al., 2014), canola meal (Gopinger et al., 2014), different protein sources (Qaisrani et al., 2014) and coarsely ground corn (Xu et al., 2015). However, no studies in relation to insect meal utilization are currently available.

4.2.2 Gut mucin composition

The entire surface of the gastrointestinal mucosa is covered by an adherent mucus gel layer that constitutes the first barrier between the intestinal lumen and absorptive cells (Forstner et al., 1995). Mucus acts as a lubricant enhancing the propulsion of chyme, modulates nutrient digestion and absorption because of its permeability, protects the underlying epithelium from physical and chemical injury and prevents the entry of enteric pathogens (Forstner and Forstner, 1994). Mucins, the main component of the mucus layer and the principal determinants of their key properties, are highly glycosylated glycoproteins that are synthesized, stored and secreted by goblet cells of the enteric epithelium (Forstner et al., 1995). The protein backbone is glycosylated by carbohydrate chains composed of different monosaccharides (Forstner and Forstner, 1994), whose chemical nature allows the histological differentiation into two broad categories: neutral and acidic, with the latter further subdivided into sialylated and sulfated mucin types (Kiernan, 1990).

The relationship between gut mucin composition and both intestinal microflora and diet has been widely investigated in broilers. Mucin monosaccharides can be affected by adhesion of gut bacteria (Gusils et al., 2003), host intestinal glycosylation (Gheri Bryk et al., 1999) and bacterial colonization (Forder et al., 2007). Simultaneously, distribution and composition of mucin carbohydrates can be also influenced by dietary modifications (Sharma et al., 1997; Fernandez et al., 2000; Smirnov et al., 2005 and 2006; Jamroz et al., 2006; Chee et al., 2010; Tsirtsikos et al., 2012a and 2012b). Several parameters, such as goblet cell (Sharma et al., 1997; Fernandez et al., 2000; Smirnov et al., 2005 and 2006; Jamroz et al., 2006; Chee et al., 2010) and mucus layer staining (Tsirtsikos et al., 2012a and 2012b), mucus layer thickness (Tsirtsikos et al., 2012a and 2012b), mucin monosaccharide composition (Tsirtsikos et al., 2012a and 2012b) and mucin gene expression (Smirnov et al., 2006;

Chee et al., 2010), have been widely investigated to evaluate the dietary effects on intestinal mucin dynamics in broilers. In particular, histochemical evaluation of goblet cells in terms of intensity staining (Sharma et al., 1997; Fernandez et al., 2000), number of positive cells (Smirnov et al., 2005 and 2006), cell density (Smirnov et al., 2005 and 2006; Chee et al., 2010) and cell size (Smirnov et al., 2005; Chee et al., 2010) has been frequently adopted as assessment method for gut mucin dynamics. Dietary modifications have been reported to widely influence intestinal mucin dynamics in broilers due to different nutrients or ingredients, such as cereal type with or without xylanase supplementation (Sharma et al., 1997; Fernandez et al., 2000), probiotics (Smirnov et al., 2005; Tsirtsikos et al., 2012b), in ovo feeding carbohydrates (Smirnov et al., 2006), plant origin active substances (Jamroz et al., 2006), manno-oligosaccharides (Chee et al., 2010) and phytogetic feed additive (Tsirtsikos et al., 2012a). However, no information regarding the insect meal utilization is currently available.

4.2.3 Gut microbiota

The gastrointestinal tract of poultry harbors a complex and dynamic microbial community (so-called microbiota) consisting primarily of anaerobic bacteria (Zhu et al., 2002). Compared with other food animals that are mammals, poultry has a shorter gastrointestinal tract and faster digesta transit, thus selecting a very different intestinal microbiota. The cecal microbiota is the most investigated intestinal microbiota in poultry (Pan and Yu, 2014), since caeca harbor the highest microbial cell densities (up to 10^{11} cells/g) and diversity, have the longest residence time (12–20 h) of digesta in the gastrointestinal tract, and are important sites for recycling of urea, water regulation, and carbohydrate fermentations contributing to intestinal health and nutrition (Ensminger, 1971; Clench and Mathias, 1995; Sergeant et al., 2014; Waite and Taylor, 2014). Direct sequencing of 16S rRNA genes is considered a powerful method to profile complex microbial communities without relying on cultivation (NRC Committee on Metagenomics, 2007), which is not able to recover the majority of microorganisms (Rappe and Giovannoni, 2003). Parallel to 16S rRNA gene-based taxonomic census data, metagenomics (and the related approaches, such as metatranscriptomics and metaproteomics) has begun to implement the understanding of microbial communities by direct sequencing of the genes (or transcripts and proteins) present in a sample, independent of cultivation biases or PCR targeting a specific gene (Handelsman, 2004).

Firmicutes, *Bacteroides* and *Proteobacteria* are the most common phyla in the chicken ceca, with *Actinobacteria* accounting for the remainder (Wise and Siragusa, 2007; Tillman et al., 2011; Wei et al., 2013; Oakley et al., 2014). Differently from phyla, at genus level there is some controversy over the predominant taxa in chicken cecal microbiota. The majority of studies reported that the most predominant genera found in chicken cecum are *Clostridium*, *Ruminococcus*, *Lactobacillus* and *Bacteroides* (Gong et al., 2007; Stanley et al., 2012; Danzeisen et al., 2011; Wei et al., 2013; Costa et al., 2017), but Callaway et al. (2009) also identified *Prevotella* as the most abundant genus.

There are extensive interactions of intestinal microbiota with host and diet, with further interactions among individual gut microbes being also identified. All these interactions can deeply influence animal nutrition and health, and are subsequently of great importance to poultry production (Pan and Yu, 2014). In relation to poultry host, the gut microbiota can be involved in the exchange of nutrients and in the modulation of host gut morphology, physiology and immunity (Pan and Yu, 2014). The different members of the gut microbiota can also show various interactions, such as competition, cooperation and antagonism. In particular, intestinal microbes may compete with each other for nutrient and attachment site and can inhibit the growth of enteric pathogens by production of bacteriostatic and bactericidal substances (Pan and Yu, 2014). Furthermore, horizontal gene transfer has been reported to be an effective mechanism which contributes to bacterial diversification and facilitates bacterial adaptation to new environments (Boto, 2010; Holmgren, 2010). Finally, dietary components and antimicrobial growth promoters may also modulate the intestinal microbiota, with probiotics being particularly able to favor the growth of beneficial bacteria (Pan and Yu, 2014). Despite diets with different cereal grains (Shakouri et al., 2009; Hammons et al., 2010), protein sources and levels (Drew et al., 2004; Sun et al., 2013), fat sources (Knarreborg et al., 2002) and feed additives (Owens et al., 2008) being reported to widely affect the chicken gut microbiota, no data related to insect meal utilization are currently available.

5 FOOD QUALITY AND SAFETY IN POULTRY

As already mentioned before, gut health has a strict relationship with animal health and performance (Kogut and Arsenault, 2016). Apart from that, gut health may have profound effects on the quality of animal feed and important implications on public health and food safety (Siragusa, 2015). Therefore, gut health could be considered a conjunction ring between the poultry farming (in terms of animal health and performance) and the consumer (in terms of meat quality and safety). The current chapter will provide some information about meat quality and safety aspects in poultry industry, with particular focus on insect meal utilization.

5.1 Meat quality

The appearance, texture, juiciness, wateriness, firmness, tenderness, odor and flavor are considered the most important and perceptible meat features that influence the initial and final quality judgment by consumers before and after purchasing a meat product (Cross et al. 1986). Furthermore, some quantifiable properties of meat such as water holding capacity, shear force, drip loss, cook loss, pH, shelf life, collagen content, protein solubility, cohesiveness, and fat binding capacity are indispensable to acquire excellent functional properties ensuring a final product of exceptional quality and profitability (Allen et al. 1998).

Nutritional quality has also been reported as one of the main determinants of meat quality in poultry (Mir et al., 2017). The major components of raw poultry meat are proteins, lipids and minerals, at proportions ranging between 18.4 and 23.4%, 1.3 and 6.0%, and 0.8 and 1.2%, respectively (Culioli et al., 2003). In particular, poultry meat is an important provider of the essential polyunsaturated fatty acids (PUFAs), especially the ω -3 fatty acids (Farrell, 2013). The amounts of these important fatty acids, particularly the long-chain polyunsaturated fatty acids (LC-PUFA), may be increased more easily in chicken meat than in other livestock meats, although negative effects on the oxidative stability may erupt (Mir et al., 2017).

Dietary changes can significantly affect meat quality in poultry (Mir et al., 2017). Till now, the impact of insect meal utilization on meat quality has been scarcely investigated in chickens. However, similar meat proximate composition, cholesterol content, sensory characteristics and off-flavours and oxidative status have been observed in broiler quails fed with defatted black soldier fly larvae meal (from 10 to 15% inclusion level) when compared with control feed. Differently, with increasing levels of dietary *Hermetia illucens* meal inclusion, the total saturated fatty acid (SFA) and total monounsaturated fatty acid (MUFA) proportions raised to the detriment of the PUFA fraction, thus worsening the healthiness of the breast meat (Cullere et al., 2017). Indeed, feedstuffs with a healthy fatty acid profile are namely low in SFA and high in PUFA, and show a favourably low n-6/n-3 ratio (Karami et al., 2013). However, as larvae are monogastric animals, a possible solution to solve this

key issue could be the modulation of the rearing substrate aiming to modify the fatty acid profile of black soldier fly. In fact, Spranghers et al. (2017) recently showed that the ether extract content of the larvae can vary greatly on varying the substrate, as well as the fatty acid profile. Furthermore, black soldier fly larvae reared on fish offal have been reported to provide a feedstuff substantially enriched in n-3 PUFA (St-Hilaire et al., 2007). However, apart from the negative fatty acid composition, Cullere et al. (2017) described increased contents of aspartic acid, glutamic acid, alanine, serine, tyrosine and threonine in breast meat of broiler quails fed with defatted black soldier fly larvae meal, thus further enhancing the biological value of the meat protein. The same authors also observed the highest Ca and the lowest P values in the meat of quails fed with the highest level of *Hermetia illucens* meal inclusion. Similarly to Cullere et al. (2017), Schiavone et al. (2017b) reported unaffected crude protein content, ether extract content, thawing loss, drip loss, pH and L*, a* and b* color values in breast meat of broiler chickens fed with black soldier fly larvae fat as a replacement of soybean oil (from 50 to 100%). The same authors also observed the increase of SFA proportion to the detriment of PUFA fraction with increasing *Hermetia illucens* inclusion rate, with MUFA content being unaffected (Schiavone et al., 2017b). In relation to other insect meals, Pieterse et al. (2014) found that the sensory profile of meat from chickens fed with a 10% *Musca domestica* larvae meal was slightly different from the control group, since the higher perception of metallic aroma and aftertaste, higher sustained juiciness and a lower mealiness (dry sensation) in the mouth.

5.2 Food safety

Meat and meat products are considered an ideal substrate supporting the growth of several spoilage and pathogenic bacteria (Mataragas et al., 2008). Indeed, pathogenic non-spore-forming/spore-forming bacteria constitute a large proportion of all foodborne illness (EFSA, 2007). The presence of these microorganisms in raw poultry is the result of their contamination from the live animal, equipment, employees and environment (Bell and Kyriakides, 2002; Gianfranceschi et al., 2003; Gudbjornsdottir et al., 2004; Reij et al., 2004; Gibbons et al., 2006). In particular, *Salmonella* and *Campylobacter* spp. represent the most common agents involved in human outbreaks of foodborne disease, with poultry meat being a significant source of infection (Mataragas et al., 2008).

Another important safety issue is represented by heavy metals. Indeed, heavy metals contamination is one of the major concerns worldwide, which influences the practical and structural integrity of an environment and may have direct and indirect effects on animal and human health. Furthermore, these contaminants may lift their residues in animal and birds tissue (Ahmed et al., 2017).

Safety issues represent a topic of great concern when insects are intended to be used in feed. Indeed, potential risks associated with the use of insect protein are represented by chemical contaminants, parasites, microbiological threats, infectious prions and allergens (Charlton et al., 2015). Among the chemical contaminants, the heavy metals cadmium, lead and mercury and the

metalloid arsenic need to be taken into account. Black soldier fly larvae have been reported to accumulate cadmium (der Fels-Klerx et al., 2016; Biancarosa et al., 2017; Purschke et al., 2017), lead (van der Fels-Klerx et al., 2016; Biancarosa et al., 2017; Purschke et al., 2017), mercury (Biancarosa et al., 2017) and arsenic (van der Fels-Klerx et al., 2016; Biancarosa et al., 2017) when reared on contaminated substrates, with concentrations often exceeding the EU limits (EC, 2002). Yellow mealworm larvae (van der Fels-Klerx et al., 2016) and housefly maggots (Charlton et al., 2015) are also able to accumulate cadmium, lead and arsenic when kept on contaminated substrates, with cadmium (Charlton et al., 2015) and arsenic (van der Fels-Klerx et al., 2016) contents exceeding the EU limits (EC, 2002). From a microbiological point of view, insects can harbor different kinds of food pathogen bacteria (Belluco et al., 2013). However, the general literature seems to indicate that insects themselves do not harbor intrinsically dangerous pathogens for humans. Indeed, all documented cases of poisoning seem to be related to contamination from the environment (van Huis, 2013). In this regard, Klunder et al. (2012) pointed out the importance of hygienic handling and correct storage when evaluating the microbiological content of reared yellow mealworm larvae and house crickets. Another important aspect to consider is that some insects (i.e., beetles, cockroaches and flies) possess the ability to act as a vector, with subsequent transmission of pathogens to farm animals (Belluco et al., 2013).

Despite these aspects being quite relevant for the consumer safety, no information regarding heavy metals accumulation or microbiological threats in meat of chickens fed with insect meal is currently available.

6 AIMS OF THE PhD PROJECT

The present PhD project aims to evaluate the effects of dietary *Tenebrio molitor* (TM) and *Hermetia illucens* (HI) meal utilization on animal health and performance, gut health and food quality and safety of different chicken strains. Different partnerships contributed to the present PhD project. The PhD main activities were represented by gut health assessment (directly performed by the PhD student) and its relationship with the animal performance and quality and safety of the obtained meat products.

7 MATERIALS AND METHODS

A total of four experimental trials were performed in the present PhD project. The Experiment 1, 2 and 3 were conducted during the first year, while the Experiment 4 was performed during the second year. The four experimental trials differ for birds, husbandry, diets, growth performance and pre-slaughter procedures, while the other activities were analogous for all the experimental trials. Therefore, the distinction between the four experiments was adopted only for the above mentioned parameters.

7.1 EXPERIMENT 1

7.1.1 Birds and husbandry

The experimental trial was supported by the University of Turin (2014-2015) and by the Regione Piemonte, Italy (PSR-PIAS no. 08000558869) and was performed in collaboration with a local poultry corporation named “O.R.A. Agricola S.r.l.” sited in Cherasco (Cuneo, Italy). The experimental protocol was designed according to the guidelines of the current European and Italian laws on the care and use of experimental animals (European Directive 86 609/EEC, put into law in Italy with D.L. 116/92). Furthermore, the experimental protocol was approved by the Ethical Committee of the Department of Veterinary Sciences of the University of Turin (Italy). A poultry house of 14 m wide × 141 m long × 4.7 m high, equipped with waterproof floor and wall, completely covered with tiles and provided with automatic ventilation system was used.

A total of 160 male broiler chicks (Ross 708) at one-day of age were randomly allotted to four dietary treatments, each consisting of 5 pens as replicates with 8 chicks per pen. Each pen was 1.20 m wide × 1.20 m long and was equipped with a feeder occupying a surface of almost 1800 cm², three nipple drinkers and rice hulls as litter. During the first 3 weeks, the animals were heated by infrared lamps to maintain the suitable temperature according to standard breeding practices (Aviagen, 2014). Lighting schedule was 23 h light: 1 h darkness until day 3 and then 18 h light: 6 hours darkness until slaughter age. At hatching, all chicks received vaccination against Newcastle disease, Gumboro disease, infectious bronchitis and coccidiosis. Vaccine recalls were performed on day 9 for infectious bronchitis and on day 18 for Gumboro and Newcastle diseases.

7.1.2 Diets

A diet based on corn meal, corn gluten meal and soybean meal was formulated and served as control (C), while 5, 10 and 15% full-fat TM larvae meal (Gaobeidian Shannong Biology CO., LTD, Gaobeidian, Hebei province, China) inclusion as a partial replacement of soybean meal, corn gluten meal and soybean oil constituted the three experimental treatment groups (TM5, TM10 and TM15) (Table 4). TM meal nutritive composition was previously analyzed as reported by De Marco et al. (2015) and summarized as follows: dry matter (DM), 948.0 (g/kg as fed); organic matter, 912.0 (g/kg as fed); crude protein (CP), 524.0 (g/kg as fed); ether extract (EE), 280.0 (g/kg as fed); neutral detergent fibre (NDF), 117.0 (g/kg as fed); acid detergent fibre (ADF), 79.5 (g/kg as fed); methionine, 5.9 (g/kg as fed); lysine, 14.7 (g/kg as fed); gross energy, 24.4 (MJ/kg DM); apparent metabolizable energy (AMEn), 16.02 (MJ/kg DM). The amount of chitin of the TM meal (46.2 g/kg as fed) was previously estimated by Bovera et al. (2016) as follows: $\text{chitin (\%)} = \text{ash free ADF (\%)} - \text{residual nitrogen in ADF} \times 6.25 (\%)$ (Finke, 2007). For each dietary treatment, diets were divided into three phases: a starter diet (days 1 to 12), a grower diet (days 12 to 25) and a finisher diet (days 25 to 53). For each phase, the experimental diets were isonitrogenous and isoenergetic and were formulated using the AMEn values for TM calculated *in vivo* for broiler chickens (De Marco et al., 2015). Diets met or exceeded NRC (1994) requirements and were adjusted according to Aviagen (2014) broiler nutrition specifications. Feed and water were provided *ad libitum*. The diets were ground to pass through a 0.5-mm sieve and stored in airtight plastic containers for DM (method number 943.01), ash (method number 924.05), CP (method number 954.01), EE (method number 920.39), NDF (method number 2002.04) and ADF (method number 973.18) determination (AOAC, 2004).

Table 4. Ingredients (g/kg as fed), apparent metabolizable energy (MJ/kg DM) and nutrient composition (%) of the 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 larvae 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
Choline chloride	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 DM)	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

¹Mineral-vitamin premix (Final B Prisma, IZA SRL, Forlì, Italy), 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.

²Calculated according to Sauvante et al. (2004) (ingredients AMEn, calcium, available phosphorus) and De Marco et al. (2015) (insect AMEn, digestible methionine, digestible lysine and digestible threonine).

TM, *Tenebrio molitor*; AMEn, apparent metabolizable energy; DM, dry matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*.

7.1.3 Growth performance

The experimental trial lasted 53 days. Health status and mortality were daily monitored during the whole experimental period. Live weight (LW) of the animals was recorded at an individual level at the beginning of the trial, at day 12, 25 and 53. Average daily feed intake (DFI) was recorded at a pen level at the end of each growth period. Feed conversion ratio (FCR) was determined for each growth period and for the overall experimental period. All measurements were made on the pen basis using a high precision electronic scale (Sartorius – Signum®).

7.1.4 Pre-slaughter procedures

At day 53, all birds were individually weighed and 10 broilers/diet (2 birds/pen) were chosen on the basis of pen average LW and identified with a shank ring.

7.2 EXPERIMENT 2

7.2.1 Birds and husbandry

The experimental trial was supported by the University of Turin (2014-2015) and by the Regione Piemonte, Italy (PSR-PIAS no. 08000558869) and was performed in collaboration with a local poultry corporation named “O.R.A. Agricola S.r.l.” sited in Cherasco (Cuneo, Italy). The experimental protocol was designed according to the guidelines of the current European and Italian laws on the care and use of experimental animals (European Directive 86 609/EEC, put into law in Italy with D.L. 116/92). Furthermore, the experimental protocol was approved by the Ethical Committee of the Department of Veterinary Sciences of the University of Turin (Italy). A poultry house of 14 m wide × 141 m long × 4.7 m high, equipped with waterproof floor and wall, completely covered by tiles and provided with automatic ventilation system was used.

A total of 160 female broiler chicks (Ross 708) at one-day of age were randomly allotted to four dietary treatments, each consisting of 5 pens as replicates with 8 chicks per pen. Each pen was 1.20 m wide × 1.20 m long and was equipped with a feeder occupying a surface of almost 1800 cm², three nipple drinkers and rice hulls as litter. During the first three weeks, the animals were heated by infrared lamps to maintain the suitable temperature according to standard breeding practices (Aviagen, 2014). Lighting schedule was 23 hours light : 1 hour darkness until day 3 and 18 h light : 6 hours darkness until slaughter age. At hatching, all chicks received vaccination against Newcastle

disease, Gumboro disease, infectious bronchitis and coccidiosis. Vaccine recalls were performed on day 9 for infectious bronchitis and on day 18 for Gumboro and Newcastle diseases.

7.2.2 Diets

A basal diet based on corn meal, corn gluten meal and soybean meal was formulated and served as control (C) group, while 50, 100 and 150 g/kg full-fat TM larvae meal (Gaobeidian Shannong Biology CO., LTD, Gaobeidian, Hebei province, China) inclusion as a partial replacement of soybean meal, corn gluten meal and soybean oil constituted the three experimental treatment groups (TM5, TM10 and TM15) (Table 5). TM meal nutritive composition and energy content were the following: dry matter, 939.0 (g/kg as fed); organic matter, 912.0 (g/kg as fed); crude protein (CP), 519.0 (g/kg as fed); ether extract (EE), 236.0 (g/kg as fed); 117.0 (g/kg as fed); neutral detergent fibre (NDF); 79.5 (g/kg as fed); acid detergent fibre (ADF); DL-methionine, 10.1 (g/kg as fed); L-lysine, 35.9 (g/kg as fed); gross energy, 24.4; apparent metabolizable energy (AMEn), 16.02 (MJ/kg DM). Three different diets were used per each dietary treatment during the three phases of growth: a starter diet (days 1 to 12), a grower diet (days 12 to 25) and a finisher diet (day 25 to 40). For each phase, the experimental diets were isonitrogenous and isoenergetic and were formulated using the AMEn values for TM calculated *in vivo* for broiler chickens (De Marco et al., 2015). Diets met or exceeded NRC (1994) requirements and were adjusted according to Aviagen (2014) broiler nutrition specifications. Feed and water were provided *ad libitum*.

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

Table 5. Ingredients (g/kg as fed), apparent metabolizable energy (MJ/kg DM) and nutrient composition (%) of the 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

¹Mineral-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.

²Calculated according to Sauvante et al. (2004) (ingredients AMEn, calcium, available phosphorus) and De Marco et al. (2015) (insect AMEn, digestible methionine, digestible lysine and digestible threonine).

TM, *Tenebrio molitor*; AME, apparent metabolizable energy; DM, dry matter; CP, crude protein; EE, ether extract; NDF, neuter detergent fiber; ADF, acid detergent fiber; C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*.

7.2.3 Growth performance

Health status and mortality were daily monitored during the whole experimental period. Live weight (LW) of the animals was recorded at an individual level at the beginning of the trial, at day 12, 25 and 40. Average daily gain (ADG) and average daily feed intake (DFI) were recorded at an individual and a pen level, respectively, at the end of each growth period. Feed conversion ratio (FCR) was determined for each growth period and for the overall experimental period. All measurements were made on the pen basis using a high precision electronic scale (Sartorius – Signum®).

7.2.4 Pre-slaughter procedures

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

7.3 EXPERIMENT 3

7.3.1 Birds and husbandry

The experimental trial 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 and December 2014, when the photoperiod was 9-10L:14-15D.

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. The chicks were vaccinated at hatching against coccidiosis, Newcastle disease and infectious bronchitis.

7.3.2 Diets

Two diets were formulated: a control diet (C), normally used by the breeder, and an experimental diet (TM7.5), 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 6). 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 diets were ground to pass through a 0.5-mm sieve and stored in airtight plastic containers for DM, ash, CP, crude fibre (AOAC, 2005) and EE (Folch et al., 1957) determination. The fatty acid composition of the control and TM diets was assessed using the method described by Alves et al. (2008). Fatty acid methyl esters were separated, identified and quantified on the basis of the chromatographic conditions reported by Renna et al. (2014). Heptadecanoic acid (C17:0) was used as the internal standard. The results were expressed in absolute values as g/kg DM.

In order to perform the AA determination, samples of the diets were prepared using a 22 h hydrolysis step in 6 HCl at 112°C under a nitrogen atmosphere. Performic acid oxidation occurred prior to acid hydrolysis for methionine and cystine. The AA in the hydrolysate was determined by means of HPLC after postcolumn derivatization, according to the procedure described by Madrid et al. (2013). Tryptophan was not determined. All the analyses were performed in duplicate.

Table 6. Ingredients and chemical composition of the experimental diets.

Ingredients (g/kg as fed)	C	TM7.5
Corn meal	720.0	720.0
Soybean meal	170.0	170.0
TM meal	-	75.0
Gluten meal	75.0	-
Vitamin-mineral premix	35.0	35.0
AME (MJ/kg)	12.18	12.22
Chemical composition (g/kg DM)		
DM	868	867
CP	169	168
Crude fat	31	50
Crude fibre	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 fatty acids (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. TM, *Tenebrio molitor*; AME, apparent metabolizable energy; DM, dry matter; CP, crude protein; C = control; TM7.5 = 7.5% inclusion level of *Tenebrio molitor*.

7.3.3 Growth performance

The experimental trial lasted 54 days. The average live weight (LW) and daily feed intake (DFI) 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 (FCR) was calculated for the 43-97 day period.

7.3.4 Pre-slaughter procedures

At day 97, all birds were individually weighed and 10 chickens/diet (2 birds/pen) were chosen on the basis of pen average LW and identified with a shank ring.

7.4 EXPERIMENT 4

7.4.1 Birds and husbandry

The experimental trial was performed in a poultry facility of the Department of Agricultural, Forest and Food Sciences (DISAFA) of the University of Turin (Italy). The poultry house was 7 m wide × 50 m long × 7 m high, equipped with waterproof floor and walls, covered completely by tiles and with an automatic ventilation system. A total of 192 male broiler chickens (Ross 308) were reared from day 1 to day 35 and assigned to four dietary treatments (6 pens/treatment and 8 birds/pen). Each pen was 1.0 m wide × 1.5 m long and was equipped with a feeder, an automatic drinker and rice hulls as bedding. During the first three weeks, the animals were heated by infrared lamps to maintain the suitable temperature according to standard breeding practices (Aviagen: Ross broiler management handbook, 2014). The lighting schedule was 18 h light: 6 h dark for the whole experimental period. At hatching, chicks received vaccination against Newcastle disease, Marek disease, infectious bronchitis and coccidiosis.

7.4.2 Diets

The diets were obtained including, as fed basis, increasing levels of partially defatted HI larvae meal (0, 5, 10 and 15%; HI0, HI5, HI10 and HI15, respectively). The diets were formulated to be isonitrogenous and isoenergetic. For each treatment, the diets were split into three phases: a starter diet (1-10 d), a grower diet (10-24 d) and a finisher diet (24-35 d). All diets were isonitrogenous to

meet or exceed the NRC (1994) requirements and were adjusted according to Aviagen (2014) broiler nutrition specifications. Feed and water were provided *ad libitum* throughout the trial. Clinical signs of illness and mortality were monitored daily throughout the experimental period. The ingredients of the experimental diets are reported in Table 7.

A partially defatted HI larvae meal obtained processing larvae reared on vegetable by-products substrate was obtained from Hermetia Deutschland GmbH & Co. KG, Baruth/Mark (Germany). The chemical analysis of HI larvae meal was performed as reported by Schiavone et al. (2017a). The partially defatted HI larvae meal contained 942 g/kg of diet of dry matter (DM), 553 g/kg DM of crude protein (CP), 180 g/kg DM of ether extract (EE), 50 g/kg DM of chitin and 24.4 MJ/kg DM of gross energy. The proximate composition of the experimental diets is shown in Table 7. Chemical analyses were carried out on three replicates of each feed sample. Feed samples were ground using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland) and analysed for CP (AOAC #984.13), crude fiber (CF; AOAC # 978.10), calcium (AOAC #927.02) and phosphorous (AOAC #965.17) content according to AOAC International (2000). EE (AOAC #2003.05) was analysed according to AOAC International (2003).

Table 7. Ingredients (g/kg as fed), apparent metabolizable energy (MJ/kg DM) and chemical composition (%) of the experimental diets.

Ingredients (g/kg as fed)	Starter period				Growing period				Finisher period			
	C	HI5	HI10	HI15	C	HI5	HI10	HI15	C	HI5	HI10	HI15
Maize meal	508.8	526.9	545.5	566.9	536.1	557.1	574.5	606.1	537.2	590.8	620.3	653.2
Soybean meal (48%CP)	345.3	299	248	193	332	277.4	230	153	300	253	178	100
HI larvae meal	0	50	100	150	0	50	100	150	0	50	100	150
Corn gluten meal	54	32	15	0	35	20	0	0	21	0	0	0
Soybean oil	46.3	45.1	43	40	59.3	56.4	54.9	48	71.2	70	63.2	56
Dicalcium phosphate	6.5	8.5	10.5	12.5	4	6	8.3	10.6	3.5	6	8.3	10.5
Calcium carbonate	17.3	16.4	15.6	14.8	16.7	15.8	14.8	14	15.1	14	13.2	12.4
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	2	2.2	2.3	2.3	1.8	1.9	2	2	1.8	1.9	1.9	1.8
L-lysine	5.1	5.2	5.4	5.8	3.8	4.1	4.2	5	3.3	3.4	4.1	5
Threonine	1.9	1.9	1.9	1.9	1.5	1.5	2	2	1.1	1.1	1.2	1.3
Trace mineral-vitamin premix ^a	8	8	8	8	5	5	5	5	5	5	5	5
Choline chloride	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	1	1	1	1	1	1	1	1	1	1	1
Total	100	100	100	100	100	100	100	100	100	100	100	100
AME ^b (MJ/kg DM)	12.56	12.56	12.56	12.56	12.98	12.98	12.98	12.98	13.4	13.4	13.4	13.4
Nutrient composition (%)												
CP	23.01	22.98	23.01	23.01	21.50	21.52	21.52	21.52	19.51	19.47	19.54	19.50
EE	7.30	7.33	7.28	7.14	8.63	8.50	8.50	8.01	9.86	9.89	9.41	8.88
CF	3.25	2.99	2.70	2.41	3.21	2.91	2.64	2.25	3.08	2.82	2.43	2.04
Calcium	0.96	0.96	0.96	0.96	0.87	0.87	0.87	0.87	0.79	0.79	0.79	0.79
Phosphorus	0.48	0.48	0.48	0.48	0.43	0.43	0.43	0.43	0.40	0.40	0.40	0.40

^aMineral-vitamin premix: vitamin A (retinyl acetate), 12,500 IU; vitamin D3 (cholecalciferol), 3000 IU; vitamin E (DL- α -tocopheryl acetate), 60 IU; vitamin K (menadione sodium bisulfite), 1.02 mg; riboflavin, 2.0 mg; pantothenate, 8.0 mg; niacin, 6 mg; piridossin, 4 mg; folic acid, 0.5 mg; biotin, 0.10 mg; tiamin, 1.0 mg; vitamin B12, 20 mg; Mn, 120 mg; Zn, 80 mg; Fe, 52 mg; Cu, 15 mg; I, 1.5 mg; Se, 0.4 mg.

^bCalculated according to INRA 2004 and Schiavone et al. (2017b).

Abbreviations: AME apparent metabolizable energy, CP crude protein, EE ether extract, CF crude fiber; C = control; HI5 = 5% inclusion level of *Hermetia illucens*; HI10 = 10% inclusion level of *Hermetia illucens*; HI15 = 15% inclusion level of *Hermetia illucens*.

7.4.3 Growth performance

During the whole experimental period (1-35 days), the live weight (LW) and feed consumption (FC) of all birds were measured weekly to calculate daily feed intake (DFI), daily weight gain (DWG) and the feed conversion ratio (FCR). All measurements were carried out on the pen basis using a high precision electronic scale (Sartorius - Signum®).

7.4.4 Pre-slaughter procedures

At day 35, all birds were individually weighed and 12 broilers/diet (2 birds/pen) were chosen on the basis of pen average LW and identified with a shank ring.

7.5 EXPERIMENTS 1-2-3-4

7.5.1 Haematological and serum parameters

Before slaughter, blood samples were collected from the identified chickens of the four experiments: 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 heterophiles 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, glucose, phosphorus, magnesium, iron, uric acid and creatinine serum concentrations were measured by means of enzymatic methods in a clinical chemistry analyzer (Screen Master Touch, Hospitex diagnostics Srl., Firenze, Italy).

7.5.2 Carcass characteristics

At the end of the four experimental trials, all selected animals were euthanized by electrical stunning and bleeding 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 liver, spleen, gizzard and abdominal fat were immediately recorded. All slaughtered carcasses were stocked in a cooling chamber (0-4 °C) for 24 h. Weights of carcass-for-grilling, breast and thighs were successively recorded. Carcass-for-grilling, breast, thighs and organs weights were also expressed as percentage of LW.

7.5.3 Organ sampling and processing

The slaughtered chickens of the four experiments were submitted to a panel of anatomopathological investigations. Intestinal segment samples (approximately 5 cm in length) of duodenum, jejunum, ileum and caecum were excised and flushed with 0.9% saline to remove all the content. The collected segments of intestine were the loop of the duodenum, the tract before Meckel's diverticulum (jejunum), the tract before the ileocolic junction (ileum) and the apex of the caeca (caecum) (Figure 4). Samples of liver, spleen, thymus, bursa of Fabricius, glandular stomach, kidney and heart were also collected. Gut segments were fixed in Carnoy's and 10% buffered formalin solutions for morphometric analysis and histopathological / histochemical investigations, respectively. The other organ samples were also fixed in 10% buffered formalin solution for histopathological examination. Tissues were routinely embedded in paraffin wax blocks, sectioned at 5 µm thickness and mounted on glass slides. Cecal content was also collected into sterile plastic tubes that were promptly refrigerated (for a maximum of 2 hours) and frozen at -80°C until DNA extraction.

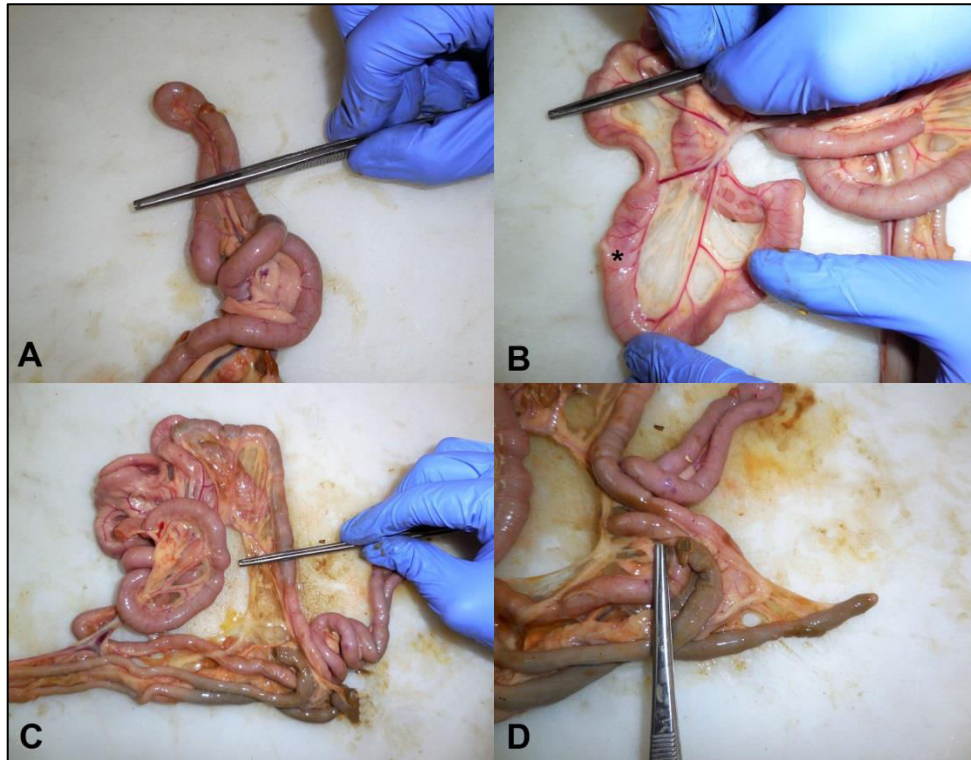


Figure 4. Intestinal segments sampled at slaughterhouse. **A)** Duodenum. **B)** Jejunum. **C)** Ileum. **D)** Caecum.

7.5.4 Histopathological examination

Formalin-fixed and paraffin-embedded organ sections from the slaughtered animals of the four experimental trials were submitted to Haematoxylin & Eosin staining and examined by light microscopy. The observed histopathological alterations were evaluated using a semiquantitative scoring system as follows: absent/minimal (score = 0), mild (score = 1) and severe (score = 2). All the slides were assessed blinded by three observers and the discordant cases were reviewed at a multi-head microscope until a consensus was reached.

7.5.5 Morphometric analysis

Carnoy-fixed and paraffin-embedded intestinal sections from the slaughtered chickens of the four experiments were submitted to Haematoxylin & Eosin staining and one slide per each intestinal segment was examined by light microscopy. Each slide was captured with a Nikon DS-Fi1 digital camera coupled to a Zeiss Axiophot microscope using a 2.5× objective lens. NIS-Elements F software was used for image capturing. Morphometric analysis was performed by Image®-Pro Plus software. 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 villus to the submucosa) and the villus height to crypt depth (Vh/Cd)

ratio (Laudadio et al., 2012) (Figure 5). Morphometric measurements were performed on 10 well-oriented and intact villi and 10 crypts chosen from duodenum, jejunum and ileum (Qaisrani et al., 2014).

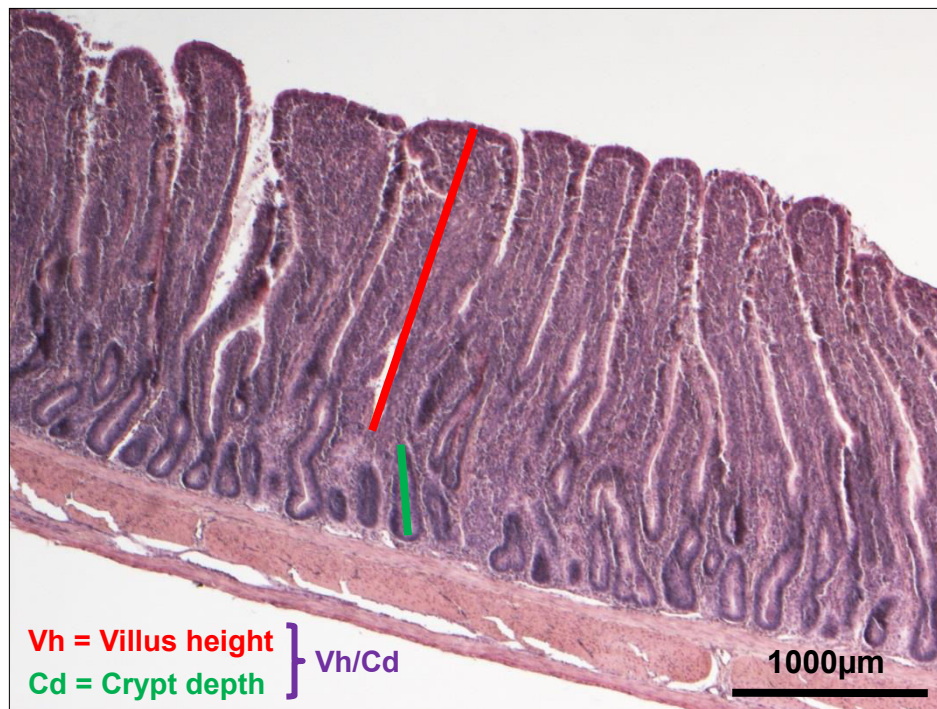


Figure 5. Morphometric analysis of small intestine. The villus height and crypt depth are directly measured, while the villus height to crypt depth ratio is subsequently obtained.

7.5.6 Histochemical staining

Formalin-fixed and paraffin-embedded intestinal sections from the slaughtered animals of the four experimental trials were submitted to three different histochemical staining, in order to characterize the three types of mucins (Forder et al., 2007).

Neutral mucins were identified by Periodic-Acid Schiff (PAS) staining. Sections were brought to water, immersed in 0.5% periodic acid solution for 20 minutes, washed in running tap water for 5 minutes and immersed in Schiff's reagent for a further 30 minutes. Sections were successively rinsed in running tap water for 10 minutes, dehydrated and mounted. Neutral mucins stained magenta (McManus, 1946).

Acidic sialylated mucins were identified by Alcian Blue, pH 2.5 (AB) staining. Sections were brought to water, immersed in 8 G X alcian blue in 3% acetic acid solution for 30 minutes, washed in running tap water for further 5 minutes and successively dehydrated and mounted. Acidic sialylated mucins stained blue (Pearse, 1960).

Acidic sulfated mucins were identified by high iron diamine (HID) staining. Sections were brought to water, oxidized in 1% periodic acid solution for 10 minutes and washed in running tap water for further

5 minutes. Sections were successively immersed in the HID solution (120 mg metadiamine, 20 mg paradiamine and 1.4 ml 10% ferric chloride in 50 ml distilled water) for 18 hours, rinsed very rapidly in tap water, dehydrated and mounted. Acidic sulfated mucins stained purple-black (Spicer, 1965). One slide per histochemical staining for each intestinal segment was examined by light microscopy. A total of 10 crypts and 10 villi for each slide were evaluated. Crypts and villi were divided into three fragments (base, midsection and tip), according to Tsirtsikos et al. (2012a and 2012b) (Figure 6A-B).

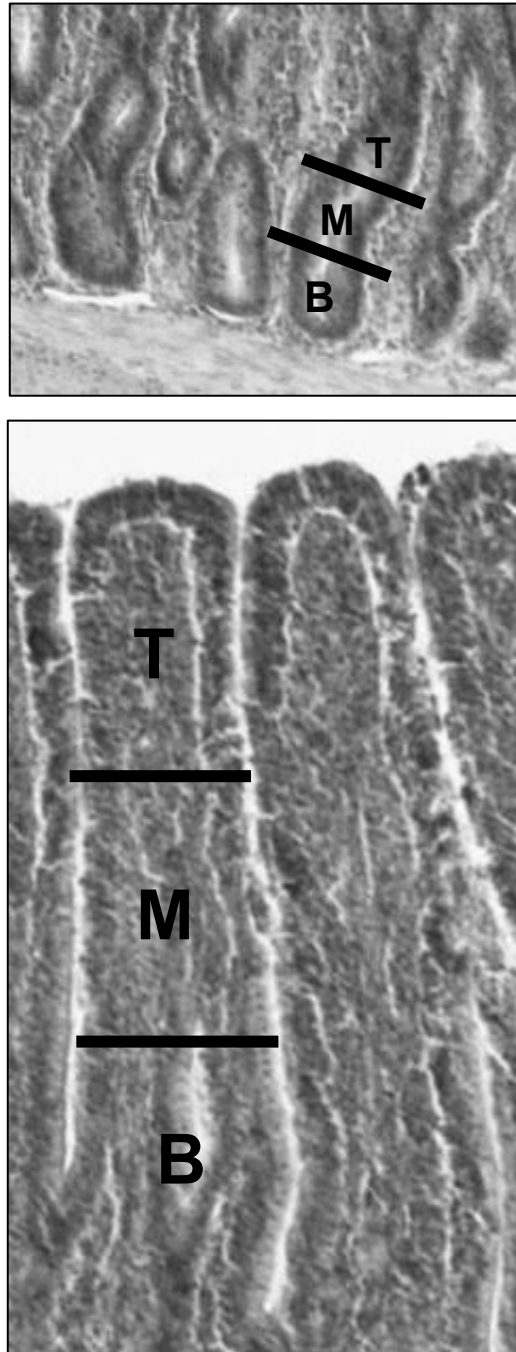


Figure 6. Schematic picture of crypt (upper photograph) and villi (lower photograph) fragments.
B = base; M = midsection; T = tip.

Mucin staining intensity of goblet cells was scored semiquantitatively for each fragment as follows: grade 0 for absent staining, grade 1 for mild staining, grade 2 for moderate staining and grade 3 for marked staining. The score was formulated according to that proposed by Tsirtsikos et al. (2012a and 2012b) for the mucus layer and depended on the number of positive goblet cells and the intensity of the staining (Figure 7-8).

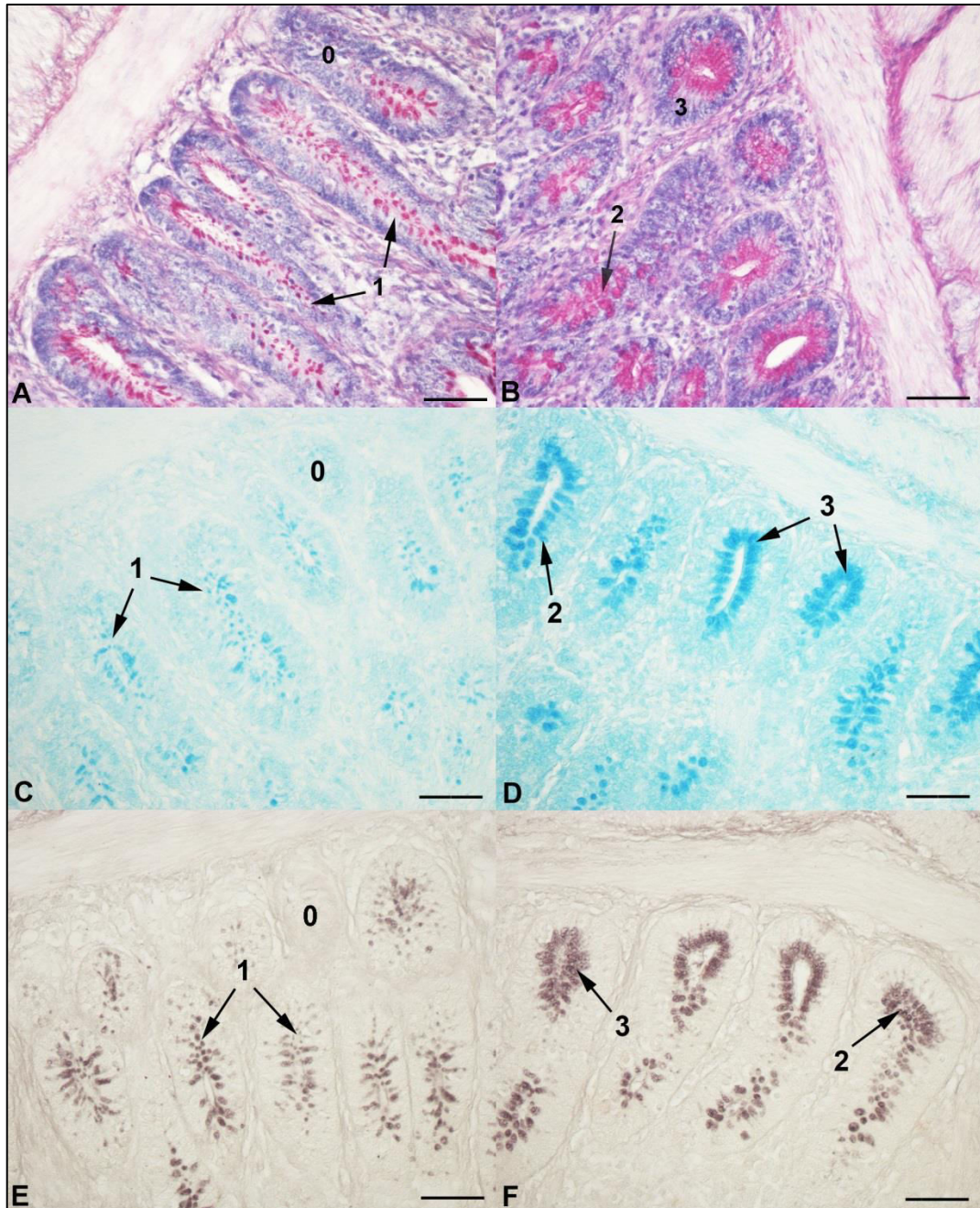


Figure 7. Mucin staining intensity scores given for crypts. **A-B)** Periodic-acid Schiff staining. **C-D)** Alcian Blue pH 2.5 staining. **E-F)** High iron diamine staining.

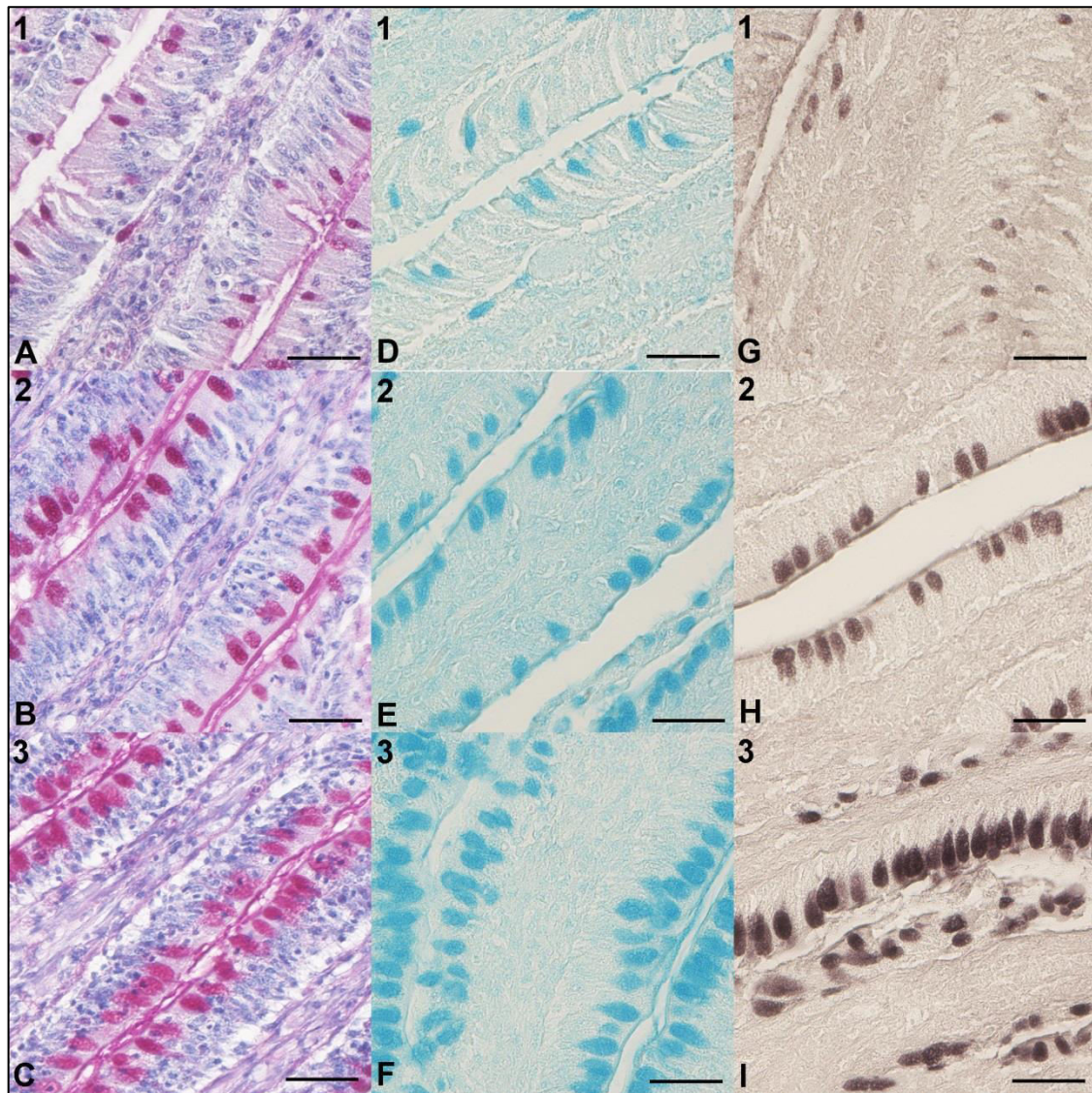


Figure 8. Mucin staining intensity scores given for villi. **A-B-C)** Periodic-acid Schiff staining, scores 1, 2 and 3. **D-E-F)** Alcian Blue pH 2.5 staining, scores 1, 2 and 3. **G-H-I)** High iron diamine staining, scores 1, 2 and 3.

All the slides were assessed blinded by three observers and the discordant cases were reviewed at a multi-head microscope until a consensus was reached.

7.5.7 DNA extraction and sequencing

The cecal content from the slaughtered chickens of all the experiments (a pool per each pen) was submitted to DNA extraction and sequencing. For the experiments 1, 2 and 3, DNA was extracted with a commercial kit (DNAzol® Reagent, Thermo Fisher Scientific) according to the manufacturer's instructions. One microliters of RNase (Illumina Inc, San Diego, CA) was added to digest RNA in the DNA samples, with an incubation of 1 h at 37°C. DNA was quantified using the NanoDrop and standardized at 5 ng/μl. Microbial diversity was studied by sequencing the amplified V3-V4 region of the 16S rRNA gene by using primers and PCR conditions previously reported (Klindworth et al.,

2013). Samples multiplexing, library purification pooling and sequencing was carried out as described in the “16S Metagenomic Sequencing Library Preparation” guide by Illumina. Libraries were sequenced by BMR genomics (Padova, Italy) on a MiSeq platform (Illumina Italy s.r.l., Milan, Italy), leading to 250bp, paired-end reads. For the experiment 4, DNA was extracted with the RNeasy Power Microbiome KIT (Qiagen, Milan, Italy) according to the manufacturer’s instructions. One μL of RNase (Illumina Inc, San Diego, CA) was added to digest RNA in the DNA samples, with an incubation of 1 h at 37°C. DNA was quantified using the NanoDrop and standardized at 5 ng/ μL . Due to poor DNA quality, one samples belonging to HI5 group was excluded. The extracted DNA was used to assess the microbiota by the amplification of the V3-V4 region of the 16S rRNA gene using the following primers: 16S-F (5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3') and 16S-R (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3') (Klindworth et al., 2013). Twenty-five μL PCR reactions were prepared using 12.5 μL of the 2X KAPA HiFi HotStart Ready Mix Taq (Kapa Biosystems, Wilmington, MA), 1 μM of each primer and 2.5 μL of DNA. 25 cycles of 30 seconds of denaturation (95 °C), 30 seconds of primer annealing (55 °C) and 30 seconds of primer elongation (72 °C), followed by a final elongation step (72 °C) of 5 minutes, were carried out. The PCR products were purified by means of an Agencourt AMPure kit (Beckman Coulter, Milan, Italy) and the resulting products were tagged by using the Nextera XT Index Kit (Illumina Inc, San Diego, CA) according to the manufacturer’s instructions. Sequencing was performed with a MiSeq Illumina instrument (Illumina) with V3 chemistry and generated 250 bp paired-end reads according to the manufacturer’s instructions. The software used for the base-calling and Illumina barcode demultiplexing processes were the MiSeq Control Soft. V2.3.0.3, the RTA v1.18.42.0 and the CASAVA v1.8.2.

7.5.8 Meat quality analysis

Technological properties (ultimate pH, color, drip and cooking losses, shear force) were assessed on the *Pectoralis major* muscle on the right breast (experimental trials 3 and 4) and on the *Biceps femoris* muscle on the right thigh (experimental trial 3) of the slaughtered chickens, while the left breast (experiments 3 and 4) and thigh (experiment 3) were frozen at -40°C until further meat chemical analysis (proximate composition and fatty acid profile).

7.5.8.1 pH

For the experimental trial 3, the pH at 24 h *post mortem* was measured on right *Pectoralis major* and right *Biceps femoris* in duplicate using a Crison portable pH-meter (Crison Instruments, S.A., Alella, Spain) fitted with a spear-type electrode and an automatic temperature compensation probe.

For the experiment 4, ultimate pH (pH_u) measurement at 48 h *post mortem* was carried out on right *Pectoralis major* using the dispersed phase method developed by Jeacocke (1977). Approximately, 2.5 g of finely minced meat were homogenized for 30 seconds by Ultraturrax in 25 ml of sodium iodoacetate (5 mM) and 18 ml of potassium chloride (150 mM) previously equilibrated at pH 7 with the aid of a solution of hydroxide of potassium (0.1 N) and one hydrochloric acid (0.1 N). The pH of the homogenate sample was subsequently determined by pH meter (Jenway 3510; Electrode 924001) previously calibrated using buffer solutions at pH 4 and 7. The values obtained were considered 2 replicates and their mean was subsequently utilized.

7.5.8.2 Color

For the experimental trial 3, meat color was measured at 24 h *postmortem* on right *Pectoralis major* and right *Biceps femoris* using a portable colorimeter Chroma Meter CR-400 Minolta (Minolta Sensing Inc., Osaka, Japan) with a 8 mm diameter measuring area, D65 illuminant and 2° standard observed. The results were expressed in terms of lightness (L^*), redness (a^*), yellowness (b^*) in the CIELAB color space (Commission Internationale de l'Éclairage, 1976). Chroma (C^*) and Hue (H^*) indexes were calculated using the following equations: $C^* = (a^{*2} + b^{*2})^{0.5}$; $H^* = \tan^{-1} (b^*/a^*)$; $H^* = 180 + \tan^{-1} (b^*/a^*)$, when $a^* < 0$. Chroma and hue describe, respectively, color saturation and taint. The color values were obtained considering the average of three readings per sample.

For the experiment 4, meat color was measured at room temperature (20°C) on inner surface of the *Pectoralis major* muscle using a portable colorimeter Chroma Meter CR-400 Konica Minolta Sensing (Minolta Sensing Inc., Osaka, Japan). Color measurements were reported in terms of lightness (L^*), redness (a^*) and yellowness (b^*) in the CIELAB color space model (Commission Internationale de l'Éclairage, 1976). The values were recorded for CIE standard illuminant D65 and the CIE 2° standard observer. The color values were obtained considering the average of three readings per sample.

7.5.8.3 Drip loss

For the experimental trial 3, the right breast and the right thigh were weighed and suspended in a covered plastic box at 4°C. After 48 hours, samples were blotted for the excess surface fluids, reweighed, and drip losses determined as percentage of weight lost by the samples during refrigerated storage period (Honikel, 1998).

For the experiment 4, a sample weighing about 80 g (approximately 7 × 4 × 3 cm) obtained from the cranial portion of each fillet was individually weighed and suspended in a plastic box at 2 to 4°C. After 48 h, samples were blotted for the excess surface fluids, reweighed and drip-loss determined as a

percentage of weight lost by the sample during the refrigerated storage period (Petracci and Baéza, 2011).

7.5.8.4 Cooking loss

For the experimental trial 4, meat samples after drip loss determination were individually weighed, packaged in a plastic bag under vacuum and cooked by immersion in a water bath (80°C) until their final internal temperature was 80°C, following the recommendations given by Petracci and Baéza (2011). Cooked samples were cooled under running water for 30 minutes. The samples were then removed from the bags, blotted and weighed. Cooking losses were determined by calculating the weight difference in samples before and after cooking and expressed as percentage of initial weight.

7.5.8.5 Allo Kramer shear force

For the experiment 4, shear values were determined using a TAHDi Heavy Duty texture analyzer (Stable Micro Systems Ltd., Godalming, Surrey, UK) equipped with an Allo-Kramer shear cell using the procedure described by Sams et al. (1990). One meat sample (approximately 2 × 4 × 1 cm) from each sample used for cook-loss determination was cut parallel with the muscle-fiber direction, weighed and sheared with the blades at a right angle to the fibers using a 250-kg load cell and cross head speed of 500 mm/min. Shear values were reported as kilograms shear per gram of sample.

7.5.8.6 Chemical composition and fatty acid profile

For the experimental trial 3, the left breast and the left thigh were cut, homogenized and divided in two parts. A portion was used to determine moisture (#950.46) and ash (#920.153) contents according to the AOAC procedures (2000). The remaining part was freeze-dried and afterwards analyzed for protein, fat and ash content, and FA composition.

The nitrogen content was determined by Kjeldahl method (# 928.08; AOAC International, 2000) and the nitrogen to protein conversion factor of 6.25 was used to calculate protein content. The intramuscular fat content was determined by Soxhlet extraction with petroleum ether according to the AOAC procedures (# 991.36; AOAC International, 2000). The results of proximate composition were expressed as percentage of fresh meat.

The FA composition was assessed as reported by Schmid et al. (2009). Briefly, total lipids were extracted with dichloromethane/methanol (2:1, v:v) by homogenization at room temperature. The solution was filtered in a separating funnel containing 1 ml of a water solution of MgCl₂ and 20 ml of

water. The organic phase was separated and evaporated to dryness. Glycerides were saponified with a solution of NaOH 0.5 M in methanol and then BF₃ (about 10% in methanol) was added for methylation. Peaks were identified by injecting pure FAME standards as detailed by Renna et al. (2012). Quantification was assessed using tridecanoic acid (C13:0) as internal standard. The results were expressed as mg/100 g of fresh meat (FM).

For the experiment 4, moisture and ashes of the left breast muscle were determined according to the Association of Official Analytical Chemists procedure (AOAC, 1990). Proteins were determined using the standard Kjeldahl copper catalyst method (AOAC, 1990). Total lipids were measured using a modification of the chloroform: methanol procedure described by Folch et al. (1957). Fatty acids (FAs) were then determined as previously reported by Glass and Cristopherson (1969). In short, 250 µg of lipids and 500 µl of the methylating solution (KOH/methanol 2 N) were put into a vial containing 5 ml of hexane and 1 g of anhydrous sodium sulfate. The vial was mixed for 30 s and placed in a water bath at 40 °C for 15 min. The sample was then stirred and cooled on ice. The upper phase, containing FAMES, was collected and used for the separation of the fatty acids using a Shimadzu GC17A gas chromatograph (Shimadzu Corporation, Tokyo, Japan) with a WP-4 Shimadzu integration system, equipped with a Varian CPSIL88 capillary column (100 m long, 0.25 mm i.d., 0.20 mm thick film) (Varian, Walnut Creek, CA, USA) and a flame ionization detector. The operating conditions of the gas chromatograph were as follows: the oven temperature was kept at 170°C for 15 min, increased to 190°C at a rate of 1 °C/min, then increased to 220°C at a rate of 5°C/min, and kept at this temperature for 17 min. The temperature of the injector and detector were 270°C and 300°C, respectively. Helium was used as the carrier gas at a constant flow rate of 1.7 mL/min. The identification of the individual FAs was carried out using PUFA-2 fatty acid methyl ester standards (Matreya, Pleasant Gap, PA, the USA), and FA quantification was obtained using methyl nonadecanoate 98% (C19:0) (Sigma, Saint Louis, the USA) as the internal standard, which was added prior to lipid extraction. The results were expressed as a percentage of each individual FAME per total FAME detected.

For both experimental trials 3 and 4, the atherogenicity (AI) and thrombogenicity (TI) indexes were calculated according to Ulbricht and Southgate (1991) as follows:

$$AI = (C12:0 + 4 \times C14:0 + C16:0) / [(\sum MUFA + \sum n-6) + \sum n-3]$$

$$TI = (C14:0 + C16:0 + C18:0) / [(0.5 \times \sum MUFA + 0.5 \times \sum n-6 + 3 \times \sum n-3) + (\sum n-3) / \sum n-6]$$

where MUFA are monounsaturated fatty acids.

7.5.9 Microbiological examination

In order to evaluate the conformity to the process hygiene criteria (EC, 2005), carcasses from the slaughtered animals of the experiment 4 were washed in peptone water and submitted to neck skin sampling for *Salmonella* spp. detection. The analyses were performed 48 hours after the slaughtering.

In parallel, in order to assess the conformity to the food safety criteria (EC, 2005), half breasts from the same animals were stored at 4° C for *Campylobacter* spp. and *Salmonella* spp. detection. The analyses were carried out 7 days after meat sectioning.

7.5.10 Heavy metals and arsenic analysis

For the experimental trial 4, chromium, iron, cobalt, nickel, copper, zinc, selenium, arsenic, lead, cadmium and mercury concentrations in HI larvae meal and breast meat were determined by the accredited test MI 351 Rev. 1/2015 (UNI EN 13804:2002, UNI EN 15763:2010 and UNI EN 13805:2014). Left breast of the slaughtered chickens (a pool per each feeding group) were previously frozen at -40°C. Aliquots of sample (0.5 g) were weighed into allotted digestion vessels and a mixture of deionized water, nitric acid and hydrogen peroxide added. The vessels were capped and the contents digested under high temperature and pressure using a single reaction chamber microwave digester system. The resulting solutions were transferred to pre-marked acid-cleaned plastic test tubes and diluted with deionized water. The metal content was determined by ICP-MS technique (inductively coupled plasma spectrometry). Multi-element measurements were made using an Agilent 7700x ICP-MS (Agilent Technologies) with collision cell.

7.5.11 Bioinformatics and statistical analysis

IBM SPSS Statistics V20.0.0 software was used to perform statistical analysis of data collected for growth performance, blood parameters, carcass characteristics, histopathological examination, morphometric analysis, histochemical investigations and meat quality parameters in all the experiments. Shapiro-Wilk's test established normality or non-normality of data distribution. χ^2 test was performed to evaluate the association between the mortality rate and the dietary treatments. For the experimental trials 1, 2 and 4, data collected for growth performance, blood parameters, carcass characteristics and meat quality parameters were tested by one-way ANOVA, evaluating the effect of dietary insect meal inclusion by polynomial contrasts. For the experiment 3, data collected for growth performance, blood parameters, carcass characteristics and meat quality parameters were tested by Student's *t* test for independent samples. Histopathological and FPD scores were analyzed by Kruskal-Wallis test (post-hoc test: Dunn's Multiple Comparison test) for the experimental trials 1, 2 and 4, while Mann-Whitney *U* test was used for the experimental trial 3. Results were expressed as mean and pooled standard error of the mean (SEM). P values < 0.05 were considered statistically significant. A statistical trend was considered for P < 0.10.

For the experiments 1, 2 and 4, intestinal morphometric indices were analyzed by fitting a general linear model. The model allowed the morphometric indices (Vh, Cd and Vh/Cd, separately) to depend

on three fixed factors (diet, intestinal segment and interaction between diet and intestinal segment). The interactions between the levels of the fixed factors were evaluated by pairwise comparisons. Results were expressed as least square mean and SEM. P values < 0.05 were considered statistically significant. A statistical trend was considered for P < 0.10. Statistical analysis was performed by procedure “General Linear Models > Univariate”. For the experiment 3, the influence of diet on intestinal morphometric measurements was tested using Student’s *t* test 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 intestinal segment within each dietary treatment. Results were expressed as mean and pooled SEM. P values < 0.05 were considered statistically significant. A statistical trend was considered for P < 0.10.

Histochemical data from all the experimental trials were analyzed by fitting a generalized linear model similar to those proposed by Tsirtsikos et al. (2012a and 2012b). The model allowed the mean mucin staining intensity scores to depend on linear predictors such as diet, mucin type, intestinal segment and fragment within crypts or villi through a negative binomial response probability distribution with a nonlinear link function (log). The bird within treatment effect was also included in the model as the repeated factor. A hybrid method for parameter estimation was used and a type III analysis with Wald chi-square test was applied to assess the model effects. Results were expressed as least squares means and SEM and the interactions between factors were evaluated by pairwise comparisons. P values < 0.05 were considered statistically significant. A statistical trend was considered for P < 0.10. Statistical analysis was performed by procedure “Generalized Linear Models”.

Paired-end reads from all the experiments were first merged using FLASH software (Magoc and Salzberg, 2011) with default parameters. Joint reads were further quality filtered (at Phred > Q20) using QIIME 1.9.0 software (Caporaso et al., 2010) and the pipeline recently described (Ferrocino et al., 2017). Briefly, operational taxonomic units (OTUs) were picked at 97% of similarity by means of UCLUST clustering methods (Edgar, 2010) and representative sequences of each cluster were used to assign taxonomy using the Greengenes 16S rRNA gene database (version 2013). Alpha diversity indices were calculated using the *diversity* function of the vegan package (Dixon, 2003). A pairwise t-test was used to assess differences between the theses. The diversity indices were further analyzed by pairwise comparisons using Wilcoxon rank sum test to assess differences between the dietary treatments. Weighted UniFrac distance matrices were used to perform Adonis and ANOSIM statistical tests in R environment. A filtered OTU table was generated at 0.1% abundance in at least 2 samples through QIIME. The table was then used to produce the Principal Component Analysis (PCA) in R environment (www.r-project.org). OTU table showed the higher taxonomy resolution reached by the 16S data. When the taxonomy assignment was not able to reach the genus level, the phylum, class, order or family were displayed. Pairwise Kruskal-Wallis tests were used to find significant differences in microbial taxa abundance according to the dietary treatment. P-values were adjusted for multiple testing and a false discovery rate (FDR) < 0.05 considered as significant.

8 RESULTS

8.1 EXPERIMENT 1

Part of the results related to the experimental trial 1 have been published (Biasato I, Gasco L, De Marco M, Renna M, Rotolo L, Dabbou S, Capucchio MT, Biasibetti E, Tarantola M, Sterpone L, Cavallarin L, Gai F, Pozzo L, Bergagna S, Dezzutto D, Zoccarato I, Schiavone A. Yellow mealworm larvae (*Tenebrio molitor*) inclusion in diets for male broiler chickens: effects on growth performance, gut morphology, and histological findings. Poult. Sci. 2018;97:540-548).

8.1.1 Growth performance

Birds remained healthy during the whole experimental period. The mortality rates of C (5%), TM5 (5%), TM10 (2.5%) and TM15 (2.5%) groups were not affected by dietary treatment ($P > 0.05$). Growth performance of the broiler chickens are summarized in Table 8. At 12 and 25 days of age, the LW increased linearly with increasing TM meal levels ($P < 0.001$ and $P < 0.05$, respectively) and the linear response increased to a maximum corresponding to the inclusion of 15 and 10% of TM meal, respectively. At 53 days of age, the LW showed quadratic response to increasing TM meal levels ($P < 0.05$), with a maximum corresponding to the inclusion of 5% of TM meal. In the period from 1 to 12 days of age, the response of DFI to the effect of TM meal inclusion was statistically significant ($P < 0.001$). In particular, the linear response increased to a maximum corresponding to the inclusion of 15% of TM meal. In the period from 12 to 25 days of age, the DFI increased linearly and quadratically with increasing TM meal levels ($P < 0.001$ and $P = 0.001$, respectively) and the linear and quadratic responses increased to a maximum corresponding to the inclusion of 15% of TM meal. On the contrary, the DFI showed no differences ($P > 0.05$) in the period from 25 to 53 days of age. In the periods from 1 to 12 and 12 to 25 days of age, the FCR was similar among the dietary treatments ($P > 0.05$). Differently, the FCR showed linear response to increasing TM meal levels in the periods from 25 to 53 ($P = 0.001$) and 1 to 53 days of age ($P < 0.05$), with a maximum corresponding to the inclusion of 15% of TM meal.

Table 8. Effect of the dietary TM larvae meal inclusion on the growth performance of the male broiler chickens.

Variable ²	Age	Dietary treatments ¹				SEM	P ³	
		C	TM5	TM10	TM15		Linear	Quadratic
LW (g)	12 d	287.00	307.58	349.72	357.98	10.12	< 0.001	0.57
	25 d	1105.10	1129.45	1272.03	1253.38	43.80	0.018	0.67
	53 d	3260.01	3641.14	3463.24	3373.07	92.76	0.73	0.037
DFI (g)	1-12 d	23.80	29.99	30.49	35.63	0.75	< 0.001	0.51
	12-25 d	81.39	111.18	104.35	112.62	2.55	< 0.001	0.001
	25-53 d	184.41	220.23	209.49	225.28	11.65	0.06	0.43
FCR (g/g)	1-12 d	1.06	1.24	1.08	1.24	0.05	0.13	0.82
	12-25 d	1.33	1.78	1.47	1.73	0.10	0.07	0.38
	25-53 d	2.23	2.54	2.68	2.97	0.12	0.001	0.93
	1-53 d	1.92	2.26	2.18	2.46	0.11	0.010	0.80

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

²LW, live weight; DFI, daily feed intake; FCR, feed conversion ratio.

³Statistical significance: P < 0.05.

8.1.2 Haematological and serum parameters

Dietary TM meal inclusion did not influence ($P > 0.05$) the haematological and serum biochemical traits of the animals (Table 9).

Table 9. Effect of the dietary TM larvae meal inclusion on the haematological and serum parameters of the male broiler chickens.

Variable ³	Dietary treatments ²				SEM	P ⁴	
	C	TM5	TM10	TM15		Linear	Quadratic
Erythrocyte (10 ⁶ cell/ μ l)	2.52	2.36	2.39	2.38	0.66	0.56	0.61
Leukocyte (10 ³ cell/ μ l)	8.12	9.06	8.34	8.03	0.45	0.82	0.53
H/L ratio	0.82	0.83	0.89	0.83	0.03	0.73	0.62
Albumin (g/dl)	1.12	1.35	0.99	1.06	0.06	0.25	0.43
Total protein (g/dl)	3.40	3.71	3.43	3.13	0.20	0.57	0.48
GGT (UI/l)	23.04	20.64	21.07	19.48	0.89	0.23	0.83
AST (UI/l)	336.17	337.37	292.75	246.86	18.01	0.06	0.51
ALT (UI/l)	9.56	11.35	10.44	11.41	0.75	0.52	0.80
Uric Acid (mg/dl)	5.85	4.12	4.85	4.32	0.33	0.20	0.36
Creatinine (mg/dl)	0.39	0.38	0.38	0.37	0.01	0.77	0.87
Triglycerides (mg/dl)	65.83	52.70	58.94	59.91	2.83	0.66	0.24
Cholesterol (mg/dl)	97.93	122.75	98.70	91.31	4.60	0.24	0.06
Glucose (mg/dl)	227.90	231.50	228.00	229.70	2.99	0.95	0.89
Phosphorus (mg/dl)	5.02	5.49	4.50	5.34	0.34	0.59	0.34
Magnesium (mEq/l)	1.49	1.15	1.02	1.32	0.10	0.30	0.80
Iron (μ g/dl)	88.97	110.28	101.37	81.62	8.12	0.48	0.81

²Four dietary treatments: C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*.

³H/L, heterophiles to lymphocytes ratio; GGT, gamma glutamyl transferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; n, number of birds.

⁴Statistical significance: P < 0.05.

8.1.3 Carcass characteristics

Dietary TM meal inclusion did not affect ($P > 0.05$) the carcass characteristics of the broiler chickens (Table 10).

Table 10. Effect of the dietary TM larvae meal inclusion on the carcass traits of the male broiler chickens.

Variable	Dietary treatments ²				SEM	P ³	
	C	TM5	TM10	TM15		Linear	Quadratic
Live weight (LW) (g)	3392	3572	3427	3323	53.17	0.47	0.20
Carcass weight (g)	2384	2489	2403	2328	41.34	0.51	0.31
Carcass weight (% LW)	70.3	69.6	70.1	70.0	0.35	0.93	0.73
Breast (g)	622	656	664	613	16.20	0.91	0.22
Breast (% LW)	18.3	18.3	19.3	18.4	0.26	0.60	0.35
Thigh (g)	743	781	730	725	12.82	0.37	0.42
Thigh (% LW)	21.9	21.8	21.3	21.8	0.21	0.71	0.53
Spleen (g)	3.93	4.00	3.62	4.39	0.20	0.59	0.41
Spleen (% LW)	0.12	0.11	0.11	0.13	0.01	0.37	0.16
Liver (g)	59.4	66.1	57.8	54.0	1.93	0.15	0.16
Liver (% LW)	1.74	1.85	1.68	1.62	0.03	0.06	0.18
Gizzard (g)	68.6	77.6	68.8	65.9	2.02	0.34	0.14
Gizzard (% LW)	2.02	2.17	2.01	1.98	0.04	0.39	0.27
Abdominal fat (g)	40.5	43.3	33.8	43.5	2.25	0.99	0.46
Abdominal fat (% LW)	1.19	1.22	1.00	1.33	0.07	0.75	0.28

²Four dietary treatments: C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*.

³Statistical significance: P < 0.05.

8.1.4 Histopathological findings

Histopathological alterations developed for spleen, thymus, bursa of Fabricius and liver and were observed in all the dietary treatments. White pulp hyperplasia or depletion was identified in the spleen (Figure 9), while cortical depletion was detected in the thymus (Figure 10). Follicular depletion, with or without intrafollicular cysts, was observed in the bursa of Fabricius (Figure 11). Finally, the liver showed different degrees of lymphoid tissue activation (Figure 12).

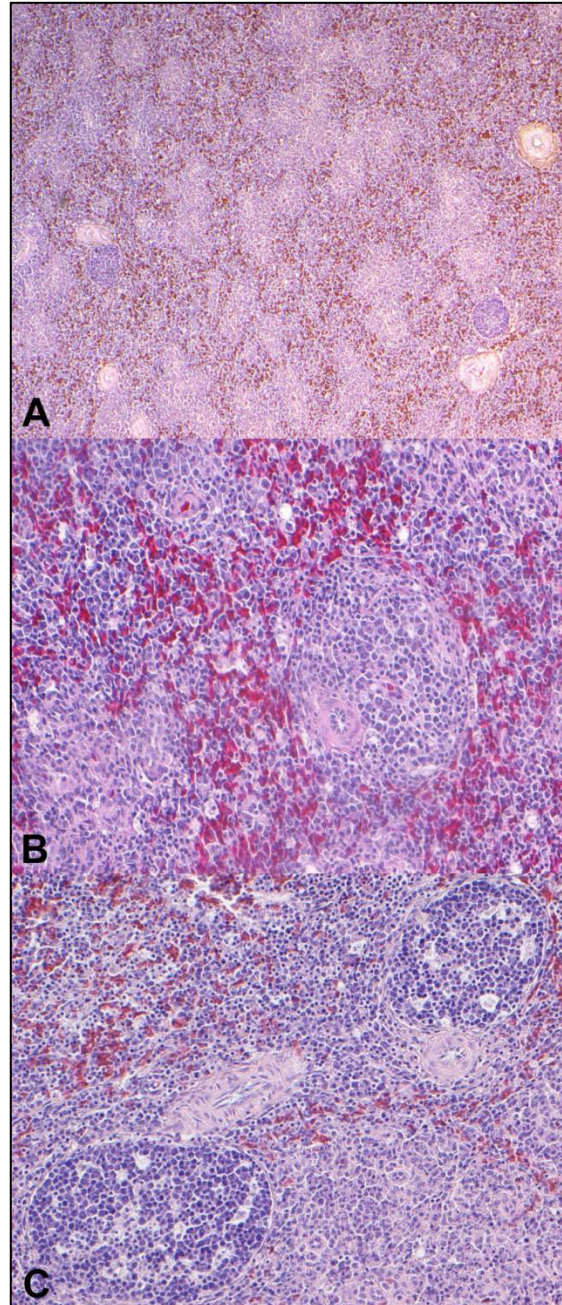


Figure 9. Histopathological alterations observed in spleen. **A)** A normal spleen (Score 0, Haematoxylin & Eosin stain, 5× magnification). **B)** Mild and multifocal white pulp depletion (Score 1, Haematoxylin & Eosin stain, 20× magnification). **C)** Severe and diffuse white pulp depletion (Score 2, Haematoxylin & Eosin stain, 20× magnification).

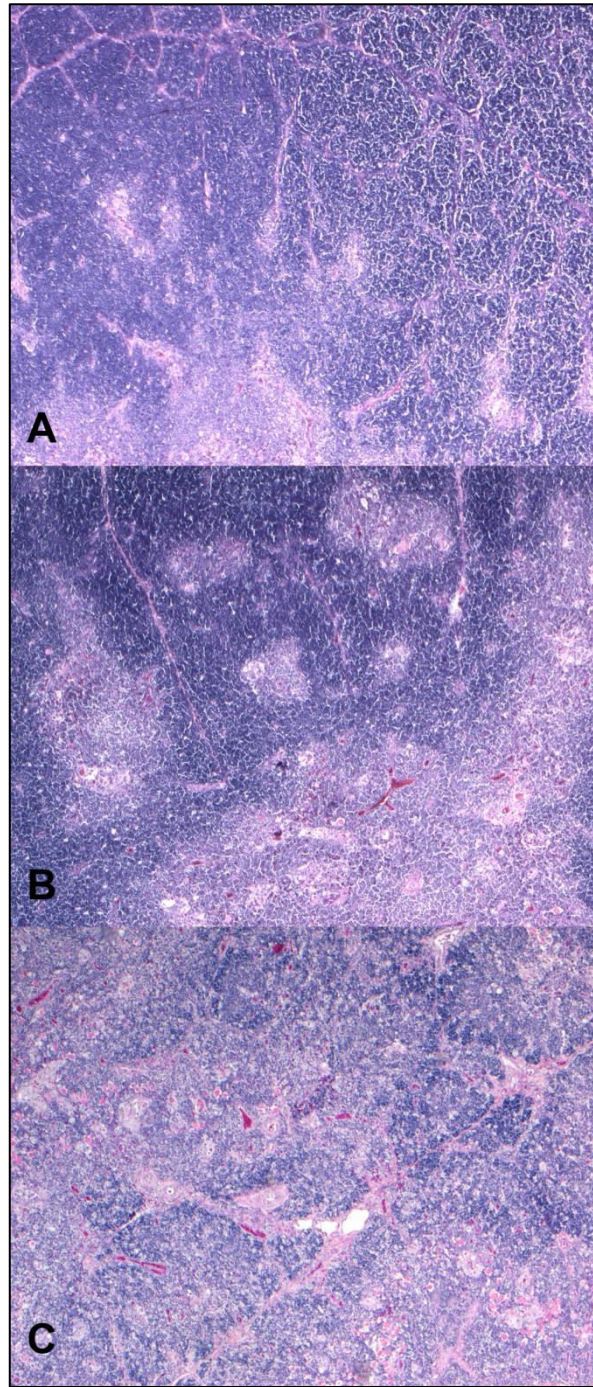


Figure 10. Histopathological alterations observed in thymus. **A)** A normal thymus (Score 0, Haematoxylin & Eosin stain, 5× magnification). **B)** Mild and multifocal cortical depletion (Score 1, Haematoxylin & Eosin stain, 5× magnification). **C)** Severe and diffuse cortical depletion (Score 2, Haematoxylin & Eosin stain, 5× magnification).

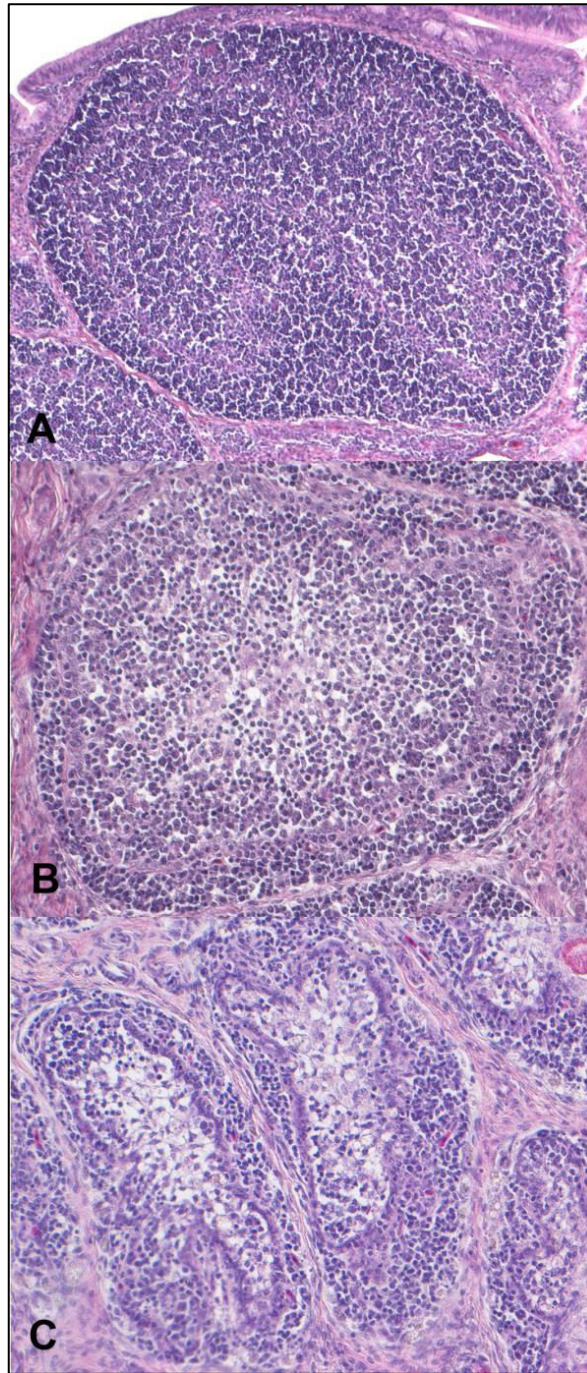


Figure 11. Histopathological alterations observed in bursa of Fabricius. **A)** A normal bursal follicle (Score 0, Haematoxylin & Eosin stain, 20× magnification). **B)** Mild and diffuse follicular depletion (Score 1, Haematoxylin & Eosin stain, 20× magnification). **C)** Severe and diffuse follicular depletion (Score 2, Haematoxylin & Eosin stain, 20× magnification).

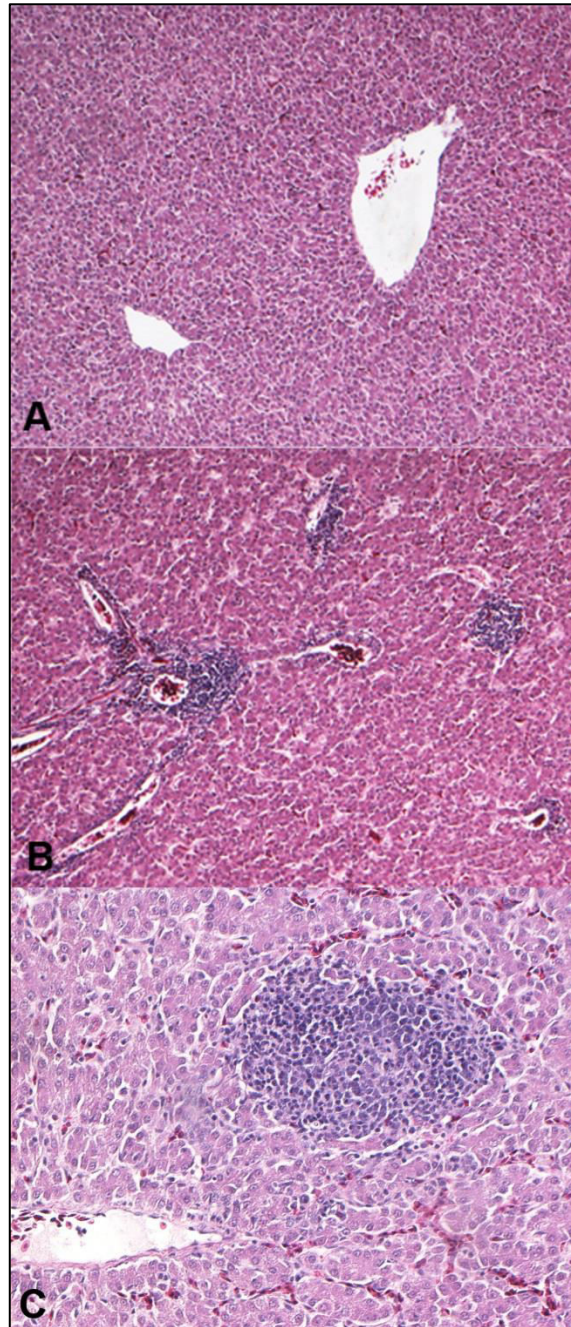


Figure 12. Histopathological alterations observed in liver. **A)** A normal liver (Score 0, Haematoxylin & Eosin stain, 10× magnification). **B)** Mild and multifocal perivascular lymphoid tissue activation (Score 1, Haematoxylin & Eosin stain, 10× magnification). **C)** Severe and focal perivascular lymphoid tissue activation (Score 2, Haematoxylin & Eosin stain, 20× magnification).

Histopathological scores of the broiler chickens are shown in Table 11. Dietary TM meal inclusion did not significantly affect ($P > 0.05$) the severity of the histopathological findings.

Table 11. Effects of dietary TM larvae meal inclusion on the histopathological scores of the male broiler chickens.

	C	TM5	TM10	TM15	SEM	P
Spleen	1.20	1.00	0.78	0.70	0.11	0.338
Thymus	0.11	0.63	0.22	0.11	0.09	0.171
Bursa of Fabricius	1.30	1.20	1.22	0.88	0.11	0.544
Liver	0.20	0.40	0.80	0.40	0.09	0.237
Intestine	No alterations					
Glandular stomach	No alterations					
Heart	No alterations					
Kidney	No alterations					

Results are expressed as mean and pooled SEM. C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*.

8.1.5 Gut mucosal morphology

The effects of the diet, intestinal segment and interaction between diet and intestinal segment on the intestinal morphometric indices are summarized in Tables 12 and 13. Vh depended on intestinal segment ($P < 0.001$) and showed a statistical trend for diet ($P = 0.093$). On the contrary, there was no influence of interaction between diet and intestinal segment ($P > 0.05$) on the morphometric indices (Table 12). In particular, duodenum and jejunum showed higher Vh ($P < 0.001$) than ileum.

Furthermore, lower Vh was found in TM15 animals ($P = 0.046$ and $P = 0.044$, respectively) compared with C and TM5 (Table 13). There was no effect of intestinal segment or interaction between diet and intestinal segment ($P > 0.05$) on Cd, while diet influenced ($P < 0.05$) the morphometric indices (Table 12). In particular, TM15 birds showed higher Cd ($P < 0.01$ and $P < 0.05$, respectively) than C and TM5 (Table 13). Vh/Cd depended on diet and intestinal segment ($P < 0.01$ and $P < 0.001$, respectively), whereas there was no influence of interaction between diet and intestinal segment ($P > 0.05$) on the morphometric indices (Table 12). In particular, TM10 and TM15 animals showed lower Vh/Cd compared with C ($P < 0.05$ and $P < 0.01$, respectively) and TM5 ($P < 0.05$ and $P < 0.01$, respectively). Furthermore, higher Vh/Cd was found in the duodenum and jejunum ($P < 0.001$) than the ileum (Table 13).

Table 12. Effects of diet, intestinal segment and interaction between diet and intestinal segment on the intestinal morphometric indices of the male broiler chickens.

Index	Fixed effect	d.f. ³	F	P ⁴
Vh (mm)	Diet ¹	3	2.194	0.093
	Intestinal segment ²	2	48.132	< 0.001
	Diet × Intestinal segment	6	1.195	0.320
Cd (mm)	Diet	3	2.875	0.040
	Intestinal segment	2	1.911	0.145
	Diet × Intestinal segment	6	0.124	0.986
Vh/Cd (mm/mm)	Diet	3	6.039	0.001
	Intestinal segment	2	15.813	< 0.001
	Diet × Intestinal segment	6	0.671	0.670

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

²Three intestinal segments: duodenum, jejunum and ileum.

³Degrees of freedom.

⁴Statistical significance: P < 0.05.

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

Table 13. Least square means of the intestinal morphometric indices in the male broiler chickens in relation to diet and intestinal segment.

Index	Fixed effect	Effect levels	Least square mean ¹	SEM
Vh (mm)	Diet ²	C	2.05 ^a	0.66
		TM5	2.06 ^a	
		TM10	1.91 ^{ab}	
		TM15	1.87 ^b	
	Intestinal segment ³	DU	2.24 ^a	0.57
		JE	2.16 ^a	
		IL	1.52 ^b	
Cd (mm)	Diet	C	0.17 ^a	0.01
		TM5	0.17 ^a	
		TM10	0.18 ^{ab}	
		TM15	0.19 ^b	
	Intestinal segment	DU	0.18	0.01
		JE	0.18	
		IL	0.17	
Vh/Cd (mm/mm)	Diet	C	12.56 ^a	0.54
		TM5	12.40 ^a	
		TM10	10.66 ^b	
		TM15	9.87 ^b	
	Intestinal segment	DU	12.70 ^a	0.47
		JE	12.19 ^a	
		IL	9.23 ^b	

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

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

³DU = duodenum; JE = jejunum; IL = ileum.

8.1.6 Gut mucin composition

Mucin staining intensity in the intestinal crypts of broiler chickens significantly depended on dietary TM meal inclusion ($P < 0.01$), mucin type ($P < 0.001$), gut segment ($P < 0.001$) and crypt fragment ($P < 0.001$) (Table 14). In particular, crypts of the TM5 animals showed higher mucin staining intensity ($P < 0.01$) compared with TM10 and TM15, but similar histochemical findings ($P > 0.05$) when compared with those of birds fed with C diet. Furthermore, greater acidic sialylated mucins staining intensity ($P < 0.001$) than neutral and acidic sulfated was found. Caecal and jejunal crypts also showed lower mucin staining intensity ($P < 0.001$) than the other gut segments and duodenum, respectively. Higher mucin staining intensity was also observed in the crypt base ($P < 0.001$) compared with the midsection and tip (Table 15, Figure 13).

Gut segment and villus fragment ($P < 0.001$) significantly influenced the mucin staining intensity in the intestinal villi, while a statistical trend was observed for mucin type ($P < 0.10$). On the contrary, there was no significant effect of dietary TM meal inclusion ($P > 0.05$) on the histochemical findings (Table 14). In particular, villi showed greater mucin staining intensity in the ileum ($P < 0.001$) than the other gut segments (Figure 14) and in the jejunum ($P < 0.001$) than the duodenum, respectively. Furthermore, lower mucin staining intensity was observed in the villus tip ($P < 0.001$) compared with the midsection and base. Villi also showed greater acidic sialylated mucin staining intensity than acidic sulfated (Table 15).

Table 14. Effects of diet, mucin type, gut segment and crypt-villus fragment on mucin staining intensity in the male broiler chickens.

Factor	d.f. ⁵	Chi-square	P ⁶
Crypts			
Diet ¹	3	12.388	0.006
Mucin type ²	2	18.860	< 0.001
Gut segment ³	3	75.407	< 0.001
Fragment ⁴	2	96.076	< 0.001
Villi			
Diet	3	4.045	0.257
Mucin type	2	4.937	0.085
Gut segment	2	748.764	< 0.001
Fragment	2	43.135	< 0.001

¹Four dietary treatments: C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*. ²Three types: neutral, acidic sialylated and acidic sulfated mucins. ³Four gut segments: duodenum, jejunum, ileum and caecum. ⁴Three fragments: base, midsection and tip. ⁵Degrees of freedom. ⁶Statistical significance: $P < 0.05$.

Table 15. Least square means of mucin staining intensity in the intestinal crypts of the male broiler chickens in relation to diet, mucin type, gut segment and crypt fragment.

	Predictor	Predictor factors	Mucin staining intensity ^{1,2}
Crypts	Diet	C	0.98 ± 0.02 ^{ab}
		TM5	1.04 ± 0.03 ^a
		TM10	0.94 ± 0.02 ^b
		TM15	0.93 ± 0.02 ^b
	Mucin type	Neutral	0.93 ± 0.02 ^b
		Acidic sialylated	1.05 ± 0.02 ^a
		Acidic sulfated	0.96 ± 0.02 ^b
	Gut segment	Duodenum	1.09 ± 0.03 ^a
		Jejunum	0.99 ± 0.02 ^b
		Ileum	1.03 ± 0.02 ^{ab}
		Caecum	0.82 ± 0.02 ^c
	Fragment	Base	1.15 ± 0.02 ^a
		Midsection	0.89 ± 0.02 ^b
		Tip	0.91 ± 0.09 ^b
Villi	Diet	C	1.70 ± 0.04
		TM5	1.69 ± 0.04
		TM10	1.59 ± 0.04
		TM15	1.67 ± 0.04
	Mucin type	Neutral	1.68 ± 0.04 ^{ab}
		Acidic sialylated	1.71 ± 0.04 ^a
		Acidic sulfated	1.60 ± 0.04 ^b
	Gut segment	Duodenum	1.01 ± 0.02 ^c
		Jejunum	1.96 ± 0.04 ^b
		Ileum	2.31 ± 0.05 ^a
	Fragment	Base	1.80 ± 0.04 ^a
		Midsection	1.71 ± 0.04 ^a
		Tip	1.49 ± 0.03 ^b

¹Data are represented as mean of counts ± SEM.

²Means with different superscript letters (a, b, c) within the same column per predictor (i.e. diet, mucin type, gut segment or fragment) differ significantly (P < 0.01).

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

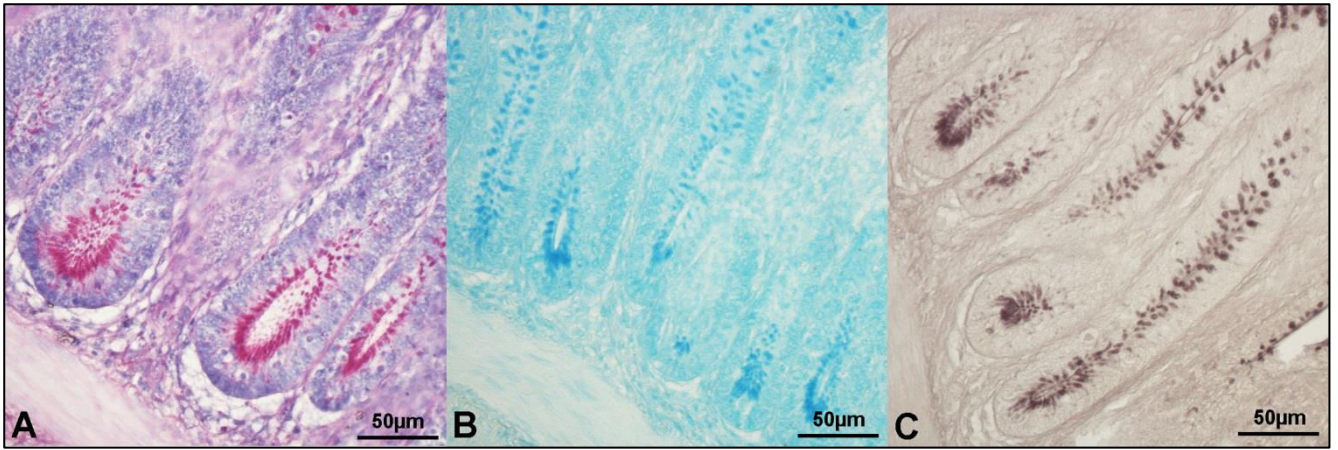


Figure 13. Histological pictures of (A) duodenal crypts stained with periodic-acid Schiff (40× magnification), (B) jejunal crypts stained with Alcian Blue pH 2.5 (40× magnification) and (C) ileal crypts stained with high iron diamine (40× magnification). Crypt bases show higher mucin staining intensity than midsection and tip fragments.

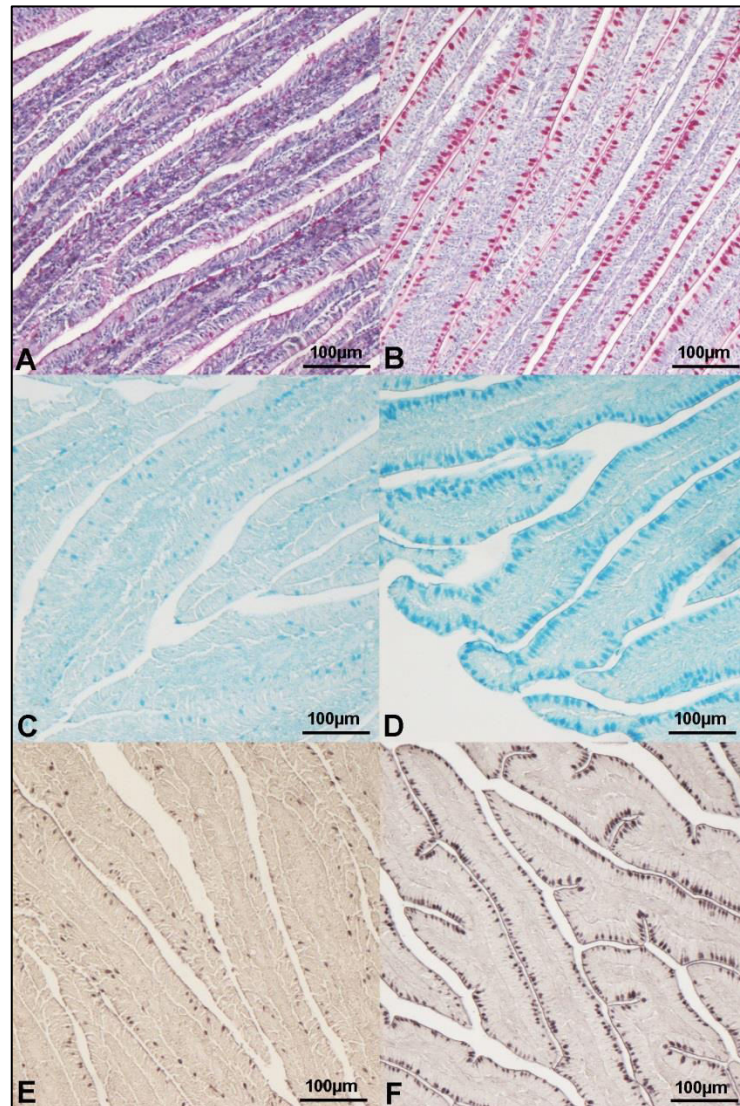


Figure 14. Histological pictures of duodenal (A, C, E) and ileal (B, D, F) villi stained with (A, B) periodic-acid Schiff (10× magnification), (C, D) Alcian Blue pH 2.5 (10× magnification) and (E, F) high iron diamine (10× magnification). Ileal villi show higher mucin staining intensity than duodenal ones.

8.1.7 Cecal microbiota characterization

A total of 2,134,649 raw reads (2x250bp) were obtained after sequencing. After joint and quality filtering, a total of 669,570 reads passed the filters applied through QIIME, with an average value of 33,479 reads/sample. In order to avoid biases due to different sequencing depths, all samples were rarefied at 3600 reads after raw read quality filtering. The rarefaction analysis and the Good's coverage (Table 16) indicated that there was a satisfactory coverage for all the samples (average Good's coverage of 86%). Moreover, Shannon index showed similar diversity ($P > 0.05$) between broiler chickens fed with TM (TM5, average value of 6.14; TM10, average value of 5.98; TM15, average value of 6.28) and C (average value of 6.34) diets (Table 16). Adonis and ANOSIM statistical tests based on Weighted UniFrac distance matrix showed significant differences among dietary treatments ($P < 0.001$). These differences were demonstrated by Principal Component Analysis (PCA), which showed a gradient of separation of the microbial membership present in each sample as a function of the diet (Figure 15).

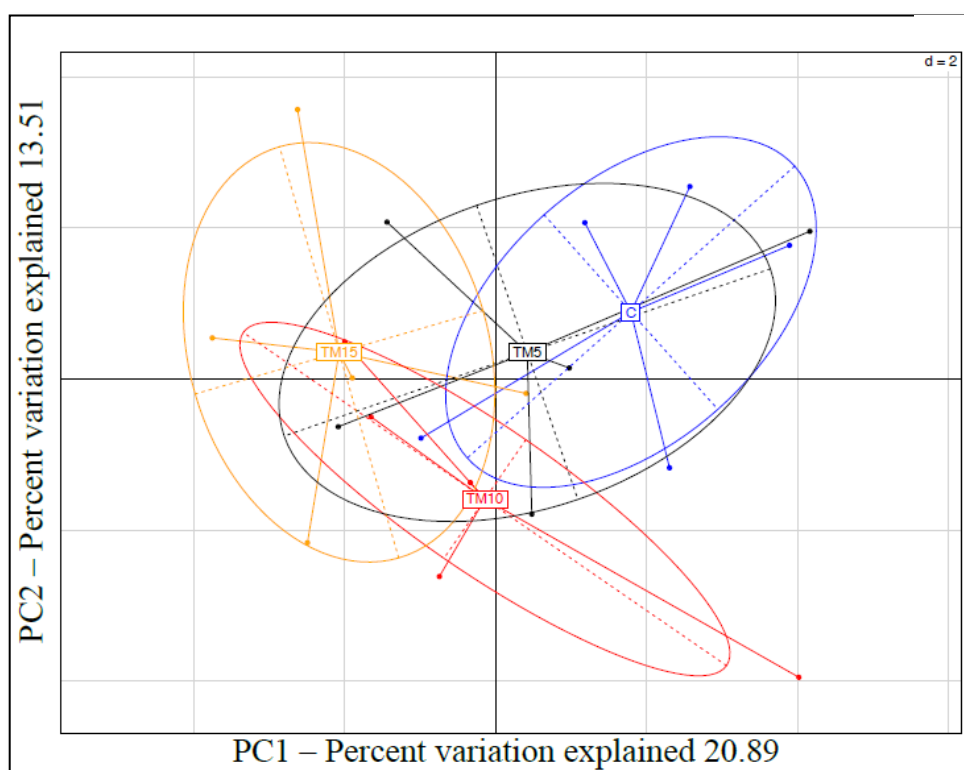


Figure 15. Bacterial community composition (weighted UniFrac beta diversity, PCA plots) in cecal samples of male broiler chickens fed with control (C), 5% (TM5), 10% (TM10) and 15% (TM15) inclusion level of *Tenebrio molitor* meal diets.

Table 16. 16S sequencing and alpha and beta diversity of the cecal microbiota of the male broiler chickens.

Diet	Description	Goods_coverage(%)	PD_whole_tree	Chao1	Observed_species	Shannon
C	M_C_1	86,73	36,42	1554,84	603	6,26
C	M_C_2	83,87	41,08	1861,82	727	6,92
C	M_C_3	86,40	36,98	1516,28	633	5,87
C	M_C_4	87,20	39,73	1459,24	614	6,39
C	M_C_5	85,17	37,80	1870,76	666	6,28
TM5	M_5_1	85,70	37,75	1991,24	621	5,86
TM5	M_5_2	85,37	39,88	1647,47	646	6,32
TM5	M_5_3	84,73	41,40	2021,13	662	6,12
TM5	M_5_4	84,80	40,77	2037,00	672	6,39
TM5	M_5_5	86,03	36,00	1626,13	631	6,03
TM10	M_10_1	86,83	37,80	1492,43	598	5,97
TM10	M_10_2	86,73	36,50	1574,45	611	6,18
TM10	M_10_3	88,40	30,63	1418,16	517	5,31
TM10	M_10_4	84,20	41,80	1958,56	699	6,47
TM10	M_10_5	86,87	39,72	1577,58	585	5,97
TM15	M_15_1	85,90	40,45	1687,04	637	6,49
TM15	M_15_2	85,43	38,78	1783,12	649	6,03
TM15	M_15_3	85,67	38,20	1960,74	624	5,91
TM15	M_15_4	84,33	44,13	1806,28	693	6,63
TM15	M_15_5	84,33	40,07	2088,01	675	6,36

Description column indicates the pool. C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*; M = male broilers.

Relative abundances of the main phyla and genera in the broiler chickens are summarized in Figure 16. *Bacteroidetes* represented the dominant bacterial phylum of the cecal community in all groups (C: 66.51%, TM5: 75.63%, TM10: 75.56% and TM15: 76.82%), outnumbering the *Firmicutes* (C: 28.01%, TM5: 21.17%, TM10: 21.58% and TM15: 19.87%, respectively) and *Proteobacteria* (C: 4.99%, TM5: 2.79%, TM10: 2.61% and TM15: 2.52%, respectively) phyla (Figure 16A). Within phylum *Bacteroidetes*, genera *Bacteroides*, *Alistipes*, *Coprobacter*, *Parabacteroides* and *Rikenella* were represented by more than 1% of the total bacterial sequences in birds fed with C (44.78%, 10.64%, 4.67%, 2.09% and 3.31%, respectively), TM5 (48.65%, 6.12%, 8.78%, 8.07% and 2.17%, respectively), TM10 (49.17%, 7.41%, 11.02%, 1.07% and 5.11%, respectively) and TM15 (47.31%, 11.86%, 7.04%, 3.24% and 4.79%, respectively) diets. *Clostridium*, *Ruminococcus*, *L-Ruminococcus* (*Ruminococcus* of *Lachnospiraceae* family), *Oscillospira* and unclassified members (U. m.) of *Lachnospiraceae* family were the predominant members of the *Firmicutes* phylum (>1% of sequences) in C (10.43%, 4.59%, 2.69%, 2.61% and 2.80%, respectively), TM5 (7.61%, 4.08%, 1.79%, 1.75% and 2.19%, respectively), TM10 (7.25%, 2.61%, 2.72%, 2.95% and 1.93%, respectively) and TM15 (7.59%, 3.27%, 1.27%, 2.57% and 1.93%, respectively) groups. Within phylum *Proteobacteria*, genus *Helicobacter* was represented by more than 1% of the total bacterial sequences in animals fed with C (4.03%), TM5 (1.77%), TM10 (1.97%) and TM15 (1.66%) diets (Figure 16B).

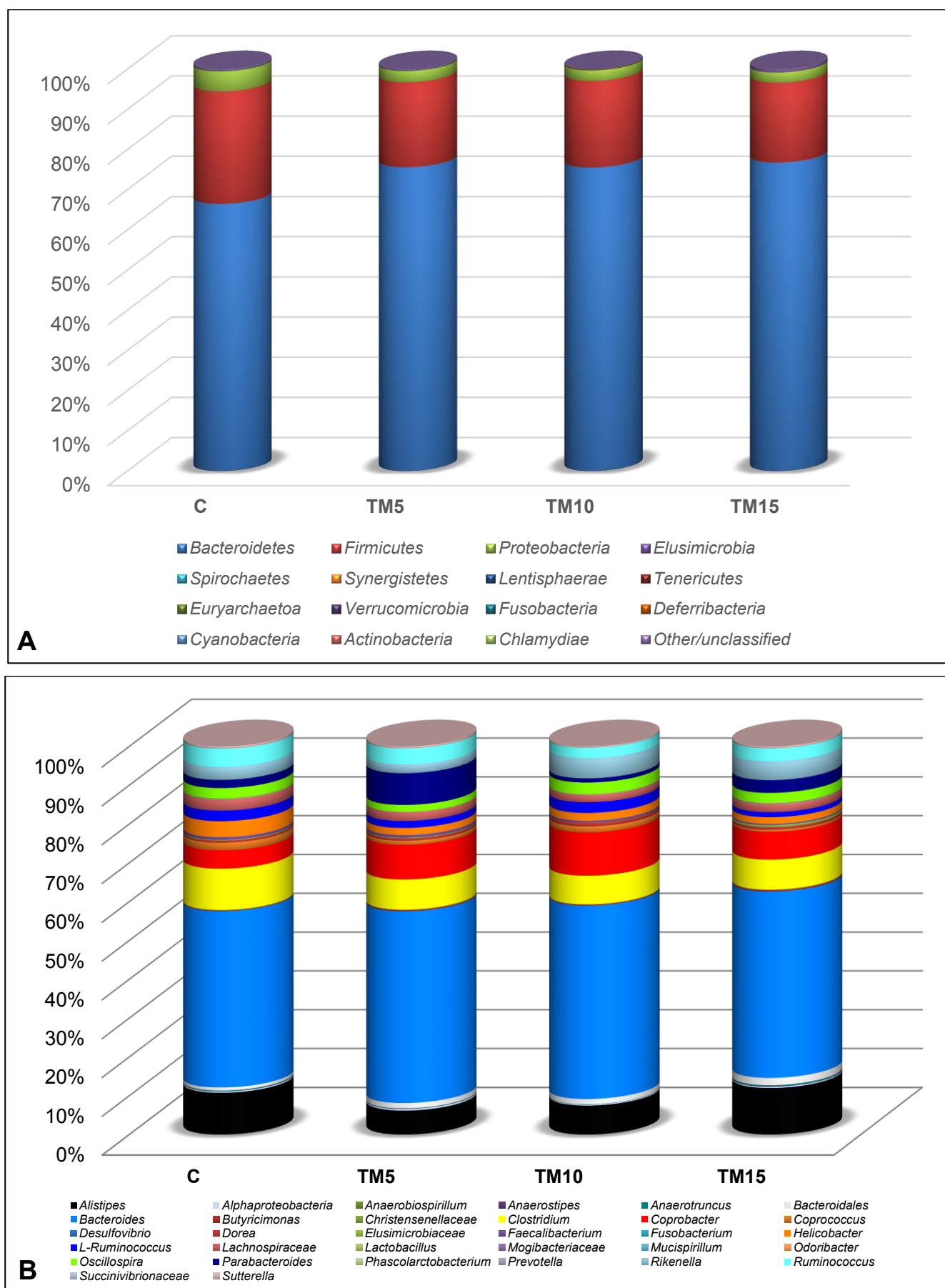


Figure 16. Relative abundance of the main bacterial phyla (A) and genera (B) in cecal samples of male broiler chickens fed with control (C), 5% (TM5), 10% (TM10) and 15% (TM15) inclusion level of *Tenebrio molitor* meal diets.

Compared to the C group, the birds fed with TM15 displayed significant decrease of the relative abundance of *Firmicutes* ($P < 0.05$, Figure 17A). On the contrary, the relative abundance of *Bacteroidetes* was unaffected by dietary TM meal inclusion ($P > 0.05$, Figure 17B). The birds fed with TM15 also showed lower *Firmicutes*:*Bacteroidetes* ratios compared to the C group ($P < 0.05$, Figure 17C).

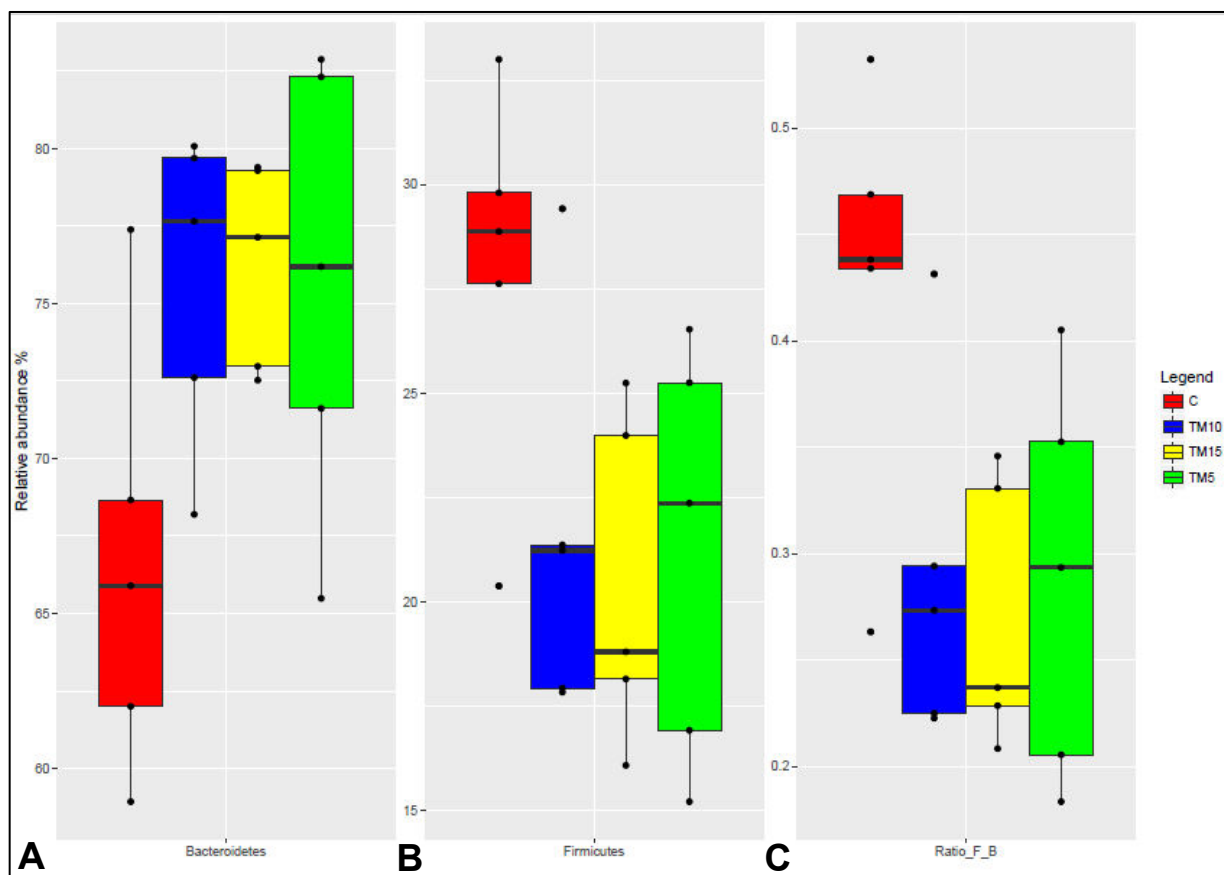


Figure 17. Boxplots of *Firmicutes* phylum (A), *Bacteroidetes* phylum (B) and *Firmicutes*:*Bacteroidetes* ratio (C) in cecal samples of male broiler chickens fed with control (C), 5% (TM5), 10% (TM10) and 15% (TM15) inclusion level of *Tenebrio molitor* meal diets. Pairwise Kruskal-Wallis test, $P < 0.05$.

At genus level, the relative abundance of *Clostridium*, *Coprococcus*, *L-Ruminococcus* and *Ruminococcus* was lower in TM animals than C ($FDR < 0.05$). The birds fed with TM10 and TM15 diets also displayed further significant decrease of the relative abundance of *Ruminococcus* compared to C and TM5 groups ($FDR < 0.05$) (Figure 18).

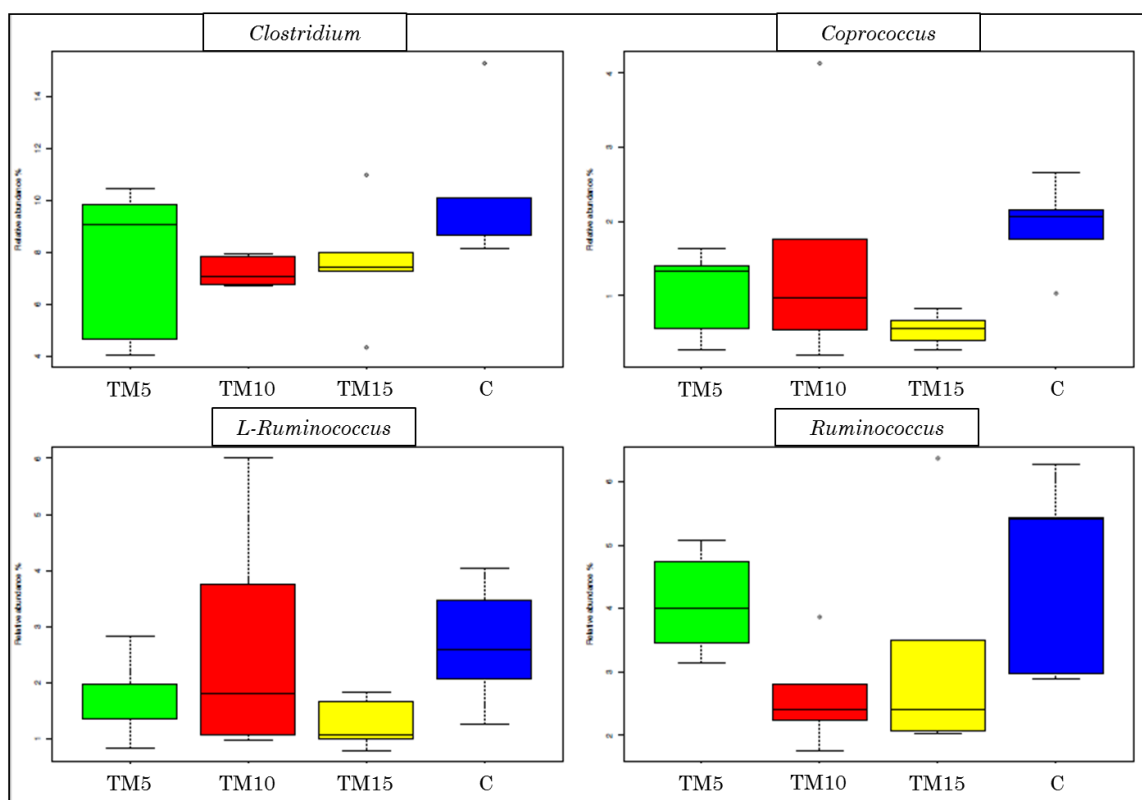


Figure 18. Boxplots of *Clostridium*, *Coprococcus*, *L-Ruminococcus* and *Ruminococcus* genera in cecal samples of male broiler chickens fed with control (C), 5% (TM5), 10% (TM10) and 15% (TM15) inclusion level of *Tenebrio molitor* meal diets. Pairwise Kruskal-Wallis test, FDR < 0.05.

8.2 EXPERIMENT 2

Part of the results related to the experimental trial 2 have been published (Biasato I, Gasco L, De Marco M, Renna M, Rotolo L, Dabbou S, Capucchio MT, Biasibetti E, Tarantola M, Bianchi C, Cavallarin L, Gai F, Pozzo L, Dezzutto D, Bergagna S, Schiavone A. Effects of yellow mealworm larvae (*Tenebrio molitor*) inclusion in diets for female broiler chickens: implications for animal health and gut histology. J. Anim. Feed. Sci. Technol. 2017;234:253-263).

8.2.1 Growth performance

No clinical signs were observed and the birds remained healthy during the whole experimental period. The mortality rates of C (2.5%), TM5 (2.5%), TM10 (0%) and TM15 (2.5%) groups were not significantly different among treatments ($P > 0.05$). Growth performance of the broiler chickens are summarized in Table 17. At 12 days of age, the LW increased linearly with increasing TM meal levels ($P < 0.05$) and the linear response increased to a maximum corresponding to the inclusion of 150 g/kg of TM meal. At 25 days of age, no significant effects related to TM meal utilization were

observed. At 40 days of age, the LW showed quadratic response to increasing TM meal levels ($P < 0.05$), with a maximum corresponding to the inclusion of 50 g/kg of TM meal. In the period from 1-12 days of age, the ADG increased linearly with increasing TM meal levels ($P < 0.05$) and the linear response increased to a maximum corresponding to the inclusion of 150 g/kg of TM meal. On the contrary, the ADG showed no differences ($P > 0.05$) in the periods from 12 to 25 and 25 to 40 days of age. In the period from 1 to 12 days of age, the response of DFI to the effect of TM meal inclusion was statistically significant ($P < 0.01$). In particular, the linear response increased to a maximum corresponding to the inclusion of 150 g/kg of TM meal. In the period from 12 to 25 days of age, the DFI increased linearly with increasing TM meal levels ($P < 0.05$) and the linear response increased to a maximum corresponding to the inclusion of 50 g/kg of TM meal. On the contrary, the DFI showed no differences ($P > 0.05$) in the period from 25 to 40 days of age. In the periods from 1 to 12, 25 to 40 and 1 to 40 days of age, the FCR was similar among the dietary treatments ($P > 0.05$). Differently, the FCR showed linear response to increasing TM meal levels in the period from 12 to 25 ($P < 0.01$), with a maximum corresponding to the inclusion of 150 g/kg of TM meal.

Table 17. Effect of the dietary TM larvae meal inclusion on the growth performance of the female broiler chickens.

Variable ²	Age	Dietary treatments ¹				SEM	P ³	
		C	TM5	TM10	TM15		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

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

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

³Statistical significance: P < 0.05.

8.2.2 Haematological and serum parameters

Haematological and serum biochemical traits of the broiler chickens are summarized in Table 18. The erythrocytes increased quadratically with increasing TM meal levels ($P < 0.05$) and the quadratic response increased to a maximum corresponding to the inclusion of 100 g/kg of TM meal. The albumin showed linear response to increasing TM meal levels ($P < 0.05$), with a minimum corresponding to the inclusion of 100 g/kg of TM meal. Similarly, the GGT decreased quadratically with increasing TM meal levels ($P < 0.05$) and the quadratic response decreased to a minimum corresponding to the inclusion of 100 g/kg of TM meal. On the contrary, no significant effects related to TM meal utilization were observed for the other blood and serum parameters ($P > 0.05$).

Table 18. Effect of the dietary TM larvae meal inclusion on the haematological and serum parameters of the female broiler chickens.

Variable ²	Dietary treatments ¹				SEM	P ³	
	C	TM5	TM10	TM15		Linear	Quadratic
Erythrocyte (10 ⁶ cell/ μ l)	2.27	2.41	2.47	2.39	0.28	0.073	0.040
Leukocyte (10 ³ cell/ μ l)	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 (U/l)	26.86	22.13	21.61	25.56	1.05	0.629	0.046
AST (U/l)	189.48	227.18	217.11	211.06	8.62	0.494	0.229
ALT (U/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 (μ g/dl)	102.44	81.20	81.26	103.89	11.64	0.985	0.392

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

²H/L, heterophiles to lymphocytes ratio; GGT, gamma glutamyl transferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

³Statistical significance: P < 0.05.

8.2.3 Carcass characteristics

Table 19 summarizes the carcass characteristics of the broiler chickens. The carcass weight increased quadratically with increasing TM meal levels ($P < 0.05$) and the quadratic response increased to a maximum corresponding to the inclusion of 50 g/kg of TM meal. The abdominal fat weight showed linear response to increasing TM meal levels ($P < 0.05$), with a maximum corresponding to the inclusion of 150 g/kg of TM meal. Similarly, the abdominal fat percentage increased linearly with increasing TM meal levels ($P < 0.01$) and the linear response increased to a maximum corresponding to the inclusion of 100 g/kg of TM meal. On the contrary, no significant effects related to TM meal utilization were observed for the other carcass traits ($P > 0.05$).

Table 19. Effect of the dietary TM larvae meal inclusion on the carcass traits of the female broiler chickens.

Variable	Dietary treatments ¹				SEM	P ²	
	C	TM5	TM10	TM15		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

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

²Statistical significance: P < 0.05.

8.2.4 Histopathological findings

Histopathological alterations developed for spleen, thymus, bursa of Fabricius and liver and were observed in all the dietary treatments. 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. Finally, the liver showed different degrees of lymphoid tissue activation. Histopathological scores of the broiler chickens are shown in Table 20. Dietary TM meal inclusion did not significantly affect ($P > 0.05$) the severity of the histopathological findings.

Table 20. Effects of dietary TM larvae meal inclusion on histopathological scores of the female broiler chickens.

	C	TM5	TM10	TM15	SEM	P
Spleen	1.50	1.20	1.10	1.10	0.13	0.705
Thymus	0.10	0.33	0.10	0.10	0.06	0.420
Bursa of Fabricius	1.90	1.88	1.22	2.00	0.05	0.778
Liver	1.00	1.10	0.90	0.30	0.12	0.056
Intestine	No alterations					
Glandular stomach	No alterations					
Heart	No alterations					
Kidney	No alterations					

Results are expressed as mean and pooled SEM. C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*.

8.2.5 Gut mucosal morphology

The effects of the diet, intestinal segment and interaction between diet and intestinal segment on gut morphometric indices of the broiler chickens are shown in Table 21 and 22. There was no influence of diet or interaction between diet and intestinal segment ($P > 0.05$) on the gut morphometric indices. On the contrary, Vh, Cd and Vh/Cd depended on intestinal segment ($P < 0.001$, $P = 0.001$ and $P < 0.001$, respectively) (Table 21). In particular, the duodenum showed higher Vh ($P < 0.001$) than the jejunum and ileum. Furthermore, higher Cd ($P < 0.001$) was found in the duodenum and jejunum than the ileum. Similarly, the duodenum and jejunum showed higher Vh/Cd ($P < 0.001$) than the ileum (Table 22).

Table 21. Effects of diet, intestinal segment and interaction between diet and intestinal segment on the intestinal morphometric indices of the female broiler chickens.

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

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

²Three intestinal segments: duodenum, jejunum and ileum.

³Degrees of freedom.

⁴Statistical significance: P < 0.05.

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

Table 22. Least square means of the intestinal morphometric indices in the female broiler chickens in relation to diet and intestinal segment.

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

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

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

³DU = duodenum; JE = jejunum; IL = ileum.

8.2.6 Gut mucin composition

Mucin staining intensity in the intestinal crypts of broiler chickens significantly depended on mucin type ($P < 0.01$), gut segment ($P < 0.001$) and crypt fragment ($P < 0.001$), while there was no significant influence of dietary TM meal inclusion ($P > 0.05$) on the histochemical findings (Table 23). In particular, crypts showed higher neutral mucins staining intensity ($P < 0.01$) than acidic sialylated and sulfated. Furthermore, lower mucin staining intensity was found in the caecal crypts ($P < 0.001$) compared with the other gut segments, with a significant increase ($P < 0.001$) also observed from the

duodenum to the ileum. Crypt base also showed greater mucin staining intensity ($P < 0.001$) than the midsection and tip (Table 24).

Dietary TM meal inclusion ($P < 0.01$), gut segment ($P < 0.001$) and villus fragment ($P < 0.05$) significantly influenced the mucin staining intensity in the intestinal villi, whereas there was no significant effect of mucin type ($P > 0.05$) on the histochemical findings (Table 23). In particular, villi of TM5 animals showed higher mucin staining intensity ($P < 0.01$) compared with TM10 and TM15, but similar histochemical findings ($P > 0.05$) when compared with those of birds fed with C diet. Differently, villi of TM10 animals showed lower mucin staining intensity ($P < 0.01$) compared with C. Furthermore, greater mucin staining intensity was observed in the jejunal and ileal villi ($P < 0.001$) than the duodenum. Villus tip also showed lower mucin staining intensity ($P < 0.05$) than the base (Table 24).

Table 23. Effects of diet, mucin type, gut segment and crypt-villus fragment on mucin staining intensity in the female broiler chickens.

Factor	d.f. ⁵	Chi-square	P ⁶
Crypts			
Diet ¹	3	3.736	0.291
Mucin type ²	2	10.084	0.006
Gut segment ³	3	216.132	< 0.001
Fragment ⁴	2	112.127	< 0.001
Villi			
Diet	3	12.569	0.006
Mucin type	2	0.762	0.683
Gut segment	2	140.155	< 0.001
Fragment	2	6.561	0.038

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

²Three types: neutral, acidic sialylated and acidic sulfated mucins.

³Four gut segments: duodenum, jejunum, ileum and caecum.

⁴Three fragments: base, midsection and tip.

⁵Degrees of freedom.

⁶Statistical significance: $P < 0.05$.

Table 24. Least square means of mucin staining intensity in the intestinal crypts of the female broiler chickens in relation to diet, mucin type, gut segment and crypt fragment.

	Predictor	Predictor factors	Mucin staining intensity ^{1,2}
Crypts	Diet	C	1.23 ± 0.03
		TM5	1.26 ± 0.03
		TM10	1.31 ± 0.03
		TM15	1.26 ± 0.03
	Mucin type	Neutral	1.33 ± 0.03 ^A
		Acidic sialylated	1.23 ± 0.02 ^B
		Acidic sulfated	1.24 ± 0.02 ^B
	Gut segment	Duodenum	1.18 ± 0.03 ^C
		Jejunum	1.40 ± 0.03 ^B
		Ileum	1.55 ± 0.03 ^A
		Caecum	1.00 ± 0.02 ^D
	Fragment	Base	1.49 ± 0.03 ^A
		Midsection	1.18 ± 0.02 ^B
		Tip	1.15 ± 0.02 ^B
Villi	Diet	C	1.82 ± 0.04 ^{AB}
		TM5	1.92 ± 0.05 ^A
		TM10	1.70 ± 0.04 ^C
		TM15	1.77 ± 0.04 ^{BC}
	Mucin type	Neutral	1.83 ± 0.04
		Acidic sialylated	1.79 ± 0.04
		Acidic sulfated	1.78 ± 0.04
	Gut segment	Duodenum	1.50 ± 0.03 ^B
		Jejunum	1.83 ± 0.04 ^A
		Ileum	2.13 ± 0.04 ^A
	Fragment	Base	1.87 ± 0.04 ^a
		Midsection	1.79 ± 0.04 ^{ab}
		Tip	1.73 ± 0.04 ^b

¹Data are represented as mean of counts ± SEM.

²Means with different superscript letters (a, b or A, B, C, D) within the same column per predictor (i.e. diet, mucin type, gut segment or fragment) differ significantly ($P < 0.05$ or $P < 0.01$, respectively).

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

8.2.7 Cecal microbiota characterization

A total of 1,985,931 raw reads (2x250bp) were obtained after sequencing. After joint and quality filtering, a total of 731,677 reads passed the filters applied through QIIME, with an average value of 36,584 reads/sample. In order to avoid biases due to different sequencing depths, all samples were rarefied at 3600 reads after raw read quality filtering. The rarefaction analysis and the Good's coverage (Table 25) indicated that there was a satisfactory coverage for all the samples (average Good's coverage of 87%). Moreover, Shannon index showed similar diversity ($P > 0.05$) between broiler chickens fed with TM (TM5, average value of 6.13; TM10, average value of 5.99; TM15, average value of 6.05) and C (average value of 6.02) diets (Table 25). Adonis and ANOSIM statistical tests based on Weighted UniFrac distance matrix showed significant differences among dietary treatments ($P < 0.001$). These differences were demonstrated by Principal Component Analysis (PCA), which showed a gradient of separation of the microbial membership present in each sample as a function of the diet (Figure 19).

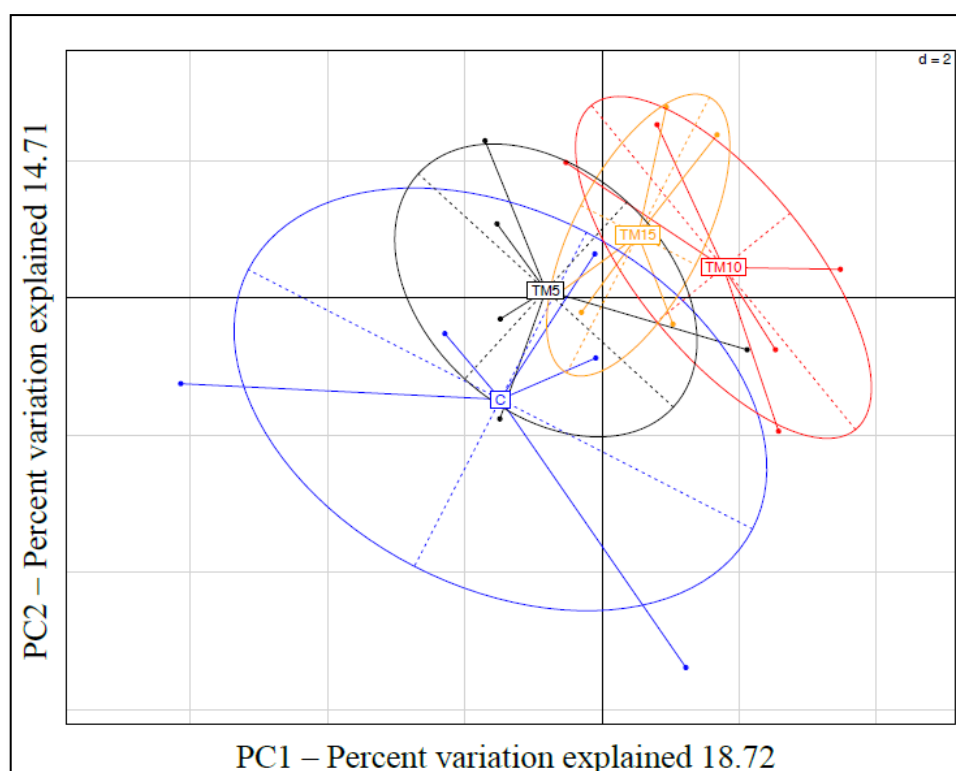


Figure 19. Bacterial community composition (weighted UniFrac beta diversity, PCA plots) in cecal samples of female broiler chickens fed with control (C), 5% (TM5), 10% (TM10) and 15% (TM15) inclusion level of *Tenebrio molitor* meal diets.

Table 25. 16S sequencing and alpha and beta diversity of the cecal microbiota of the female broiler chickens.

Diet	Description	Goods_coverage(%)	PD_whole_tree	Chao1	Observed_species	Shannon
C	F_C_1	86,73	38,58	1711,72	599	6,32
C	F_C_2	88,77	31,32	1391,63	507	5,26
C	F_C_3	87,77	35,73	1410,51	571	6,24
C	F_C_4	87,57	35,55	1797,42	536	5,16
C	F_C_5	82,20	43,76	2277,95	764	7,11
TM5	F_5_1	87,57	37,40	1391,72	585	6,19
TM5	F_5_2	89,30	33,75	1137,80	519	5,91
TM5	F_5_3	84,83	41,29	1803,66	681	6,69
TM5	F_5_4	87,43	37,31	1594,29	552	5,65
TM5	F_5_5	86,20	38,69	1758,88	619	6,21
TM10	F_10_1	89,23	33,42	1187,36	512	5,65
TM10	F_10_2	85,90	39,86	1684,04	634	6,37
TM10	F_10_3	87,37	33,71	1544,25	563	6,07
TM10	F_10_4	89,13	34,18	1359,44	491	5,85
TM10	F_10_5	86,00	39,47	1738,08	610	6,03
TM15	F_15_1	87,50	37,26	1500,70	578	6,30
TM15	F_15_2	86,80	37,21	1807,03	585	6,07
TM15	F_15_3	88,17	33,43	1552,08	522	5,56
TM15	F_15_4	86,80	39,52	1501,97	603	6,25
TM15	F_15_5	88,73	31,24	1618,73	502	6,08

Description column indicates the pool. C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*; F = female broilers.

Relative abundances of the main phyla and genera in the broiler chickens are summarized in Figure 20. *Bacteroidetes* represented the dominant bacterial phylum of the cecal community in all groups (C: 50.11%, TM5: 47.93%, TM10: 53.11% and TM15: 52.60%), outnumbering the *Firmicutes* (C: 32.46%, TM5: 31.79%, TM10: 24.50% and TM15: 27.43%, respectively) and *Proteobacteria* (C: 17.19%, TM5: 19.77%, TM10: 22.15% and TM15: 19.68%, respectively) phyla (Figure 20A). Within phylum *Bacteroidetes*, genera *Bacteroides*, *Alistipes*, *Coprobacter* and *Parabacteroides* were represented by more than 1% of the total bacterial sequences in birds fed with C (42.21%, 3.85%, 2.45% and 1.14%, respectively), TM5 (35.05%, 6.69%, 4.25% and 1.08%, respectively), TM10 (36.88%, 3.99%, 4.35%, and 6.64%, respectively) and TM15 (34.47%, 5.17%, 7.08% and 4.13%, respectively) diets. *Clostridium*, *Ruminococcus*, *L-Ruminococcus*, *Oscillospira* and unclassified members (U. m.) of *Lachnospiraceae* family were the predominant members of the *Firmicutes* phylum (>1% of sequences) in C (11.57%, 5.17%, 3.93%, 4.15% and 2.68%, respectively), TM5 (14.09%, 4.95%, 2.43%, 3.63% and 1.76%, respectively), TM10 (9.39%, 3.68%, 1.87%, 3.28% and 2.12%, respectively) and TM15 (10.47%, 3.81%, 4.27%, 2.89% and 2.15%, respectively) groups. Within phylum *Proteobacteria*, genus *Helicobacter* was represented by more than 1% of the total bacterial sequences in animals fed with C (15.94%), TM5 (17.35%), TM10 (20.14%) and TM15 (17.80%) diets (Figure 20B).

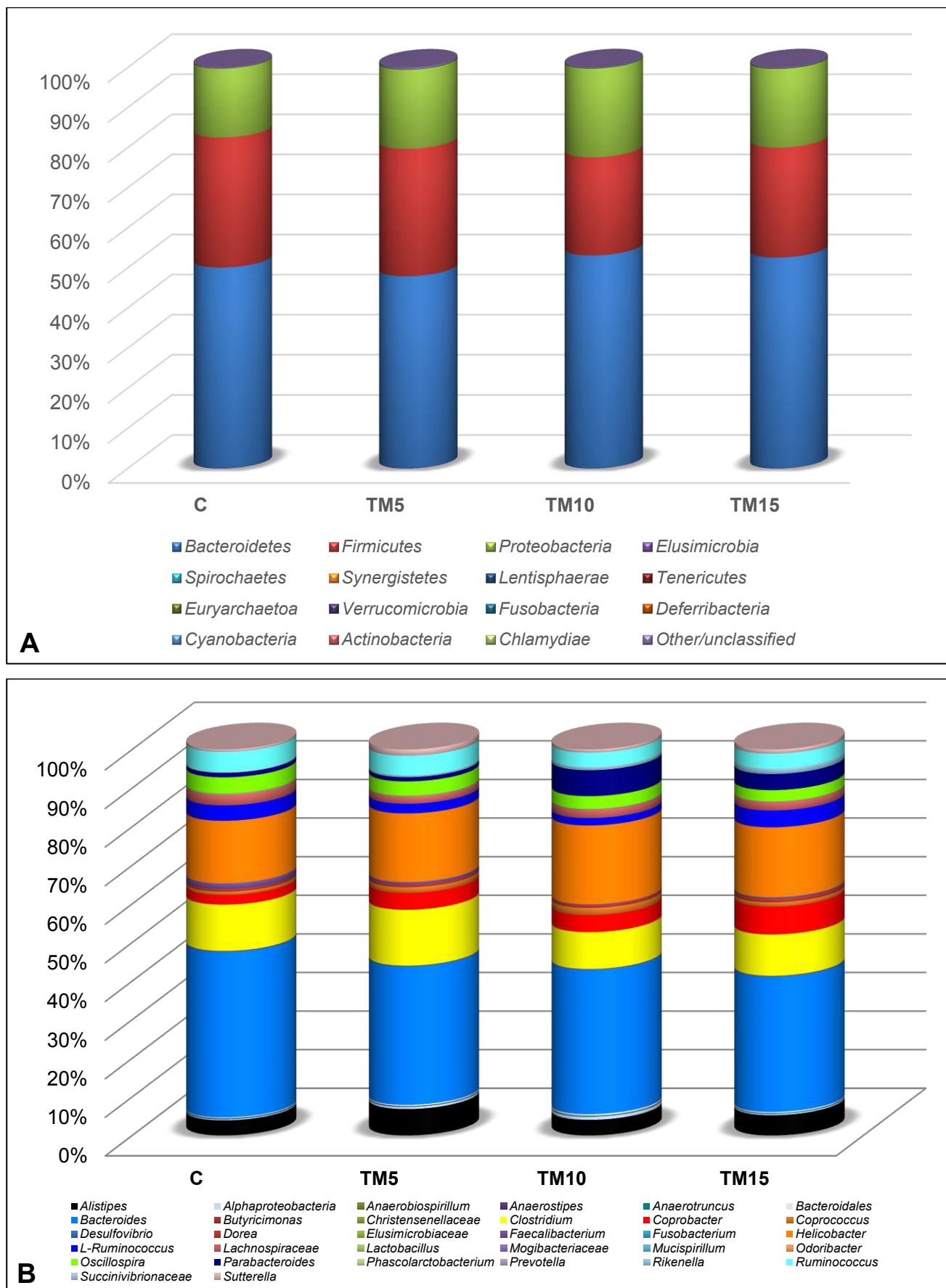


Figure 20. Relative abundance of the main bacterial phyla (A) and genera (B) in cecal samples of female broiler chickens fed with control (C), 5% (TM5), 10% (TM10) and 15% (TM15) inclusion level of *Tenebrio molitor* meal diets.

Compared to the TM5 group, the birds fed with TM10 displayed significant decrease of the relative abundance of *Firmicutes* ($P < 0.05$, Figure 21A). On the contrary, the relative abundance of *Bacteroidetes* was unaffected by dietary TM meal inclusion ($P > 0.05$, Figure 21B). The birds fed with TM15 also showed lower *Firmicutes*:*Bacteroidetes* ratios compared to the TM5 group ($P < 0.05$, Figure 21C).

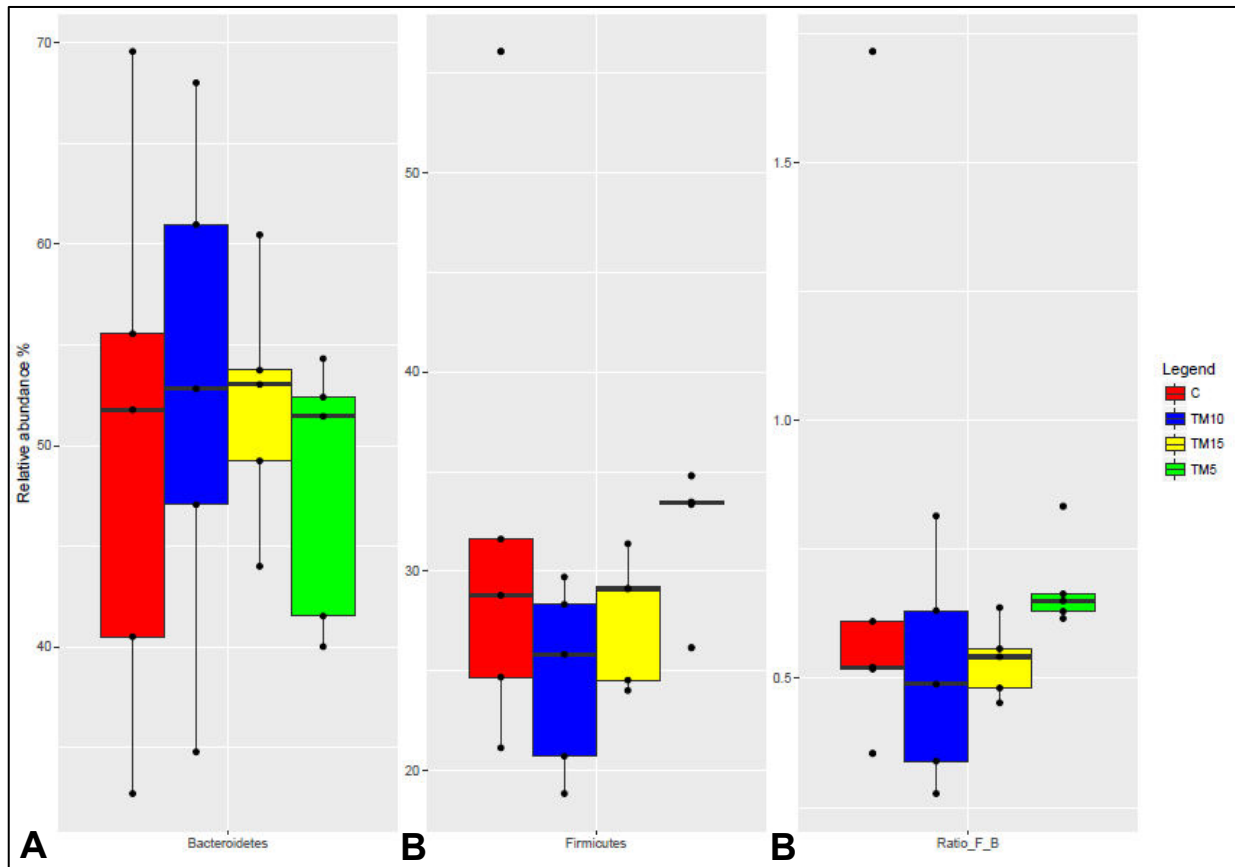


Figure 21. Boxplots of *Firmicutes* phylum (A), *Bacteroidetes* phylum (B) and *Firmicutes*:*Bacteroidetes* ratio (C) in cecal samples of female broiler chickens fed with control (C), 5% (TM5), 10% (TM10) and 15% (TM15) inclusion level of *Tenebrio molitor* meal diets. Pairwise Kruskal-Wallis test, $P < 0.05$.

At genus level, the relative abundance of *Sutterella*, *Clostridium* and *Alistipes* was higher in TM animals than C ($FDR < 0.05$). Furthermore, the birds fed with TM diets displayed significant decrease of the relative abundance of *Ruminococcus* compared to C group ($FDR < 0.05$) (Figure 22).

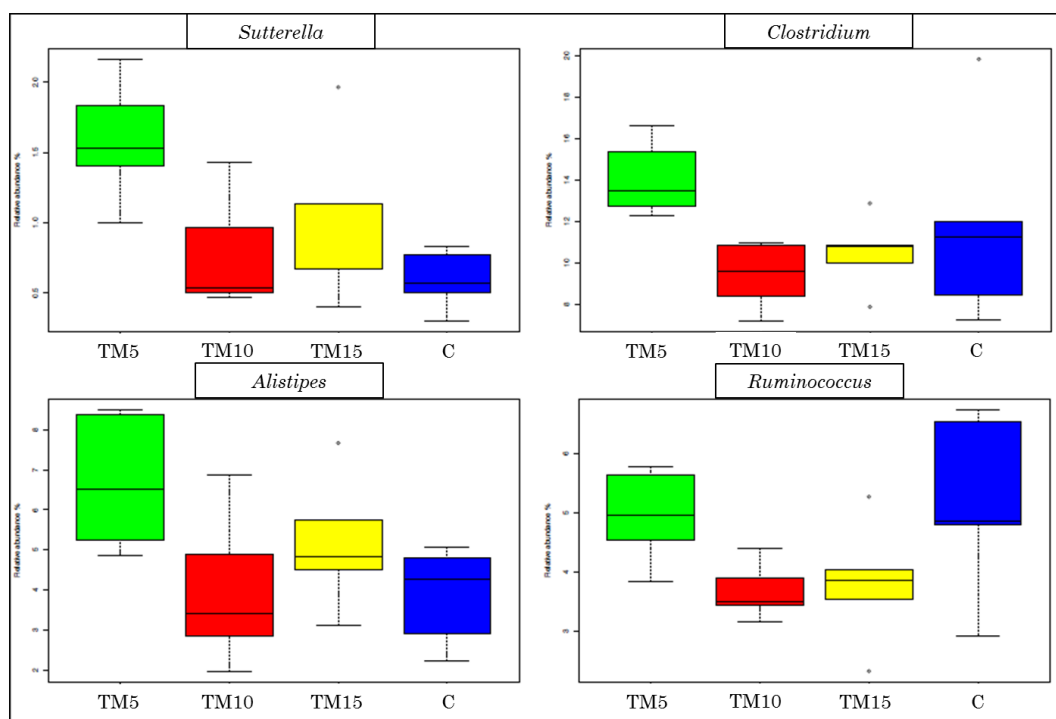


Figure 22. Boxplots of *Sutterella*, *Clostridium*, *Alistipes* and *Ruminococcus* genera in cecal samples of female broiler chickens fed with control (C), 5% (TM5), 10% (TM10) and 15% (TM15) inclusion level of *Tenebrio molitor* meal diets. Pairwise Kruskal-Wallis test, FDR < 0.05.

8.3 EXPERIMENT 3

Part of the results related to the experimental trial 3 have been published (Biasato I, De Marco M, Rotolo L, Renna M, Lussiana C, Dabbou S, Capucchio MT, Biasibetti E, Costa P, Gai F, Pozzo L, Dezzutto D, Bergagna S, Martínez S, Tarantola M, Gasco L, Schiavone A. 2016. Effects of dietary *Tenebrio molitor* meal inclusion in free-range chickens. J. Anim. Physiol. Anim. Nutr. 2016,100:1104-1112).

8.3.1 Growth performance

Dietary TM meal inclusion did not influence ($P > 0.05$) the growth performance of the animals (Table 26).

Table 26. Effect of the dietary TM larvae meal inclusion on the growth performance of the female free-range chickens.

	C	TM7.5	SEM	P
Initial body weight (d 43) (g)	720.2	712.3	7.1	0.612
Final body weight (d 97) (g)	2131.0	2162.5	70.7	0.845
Average daily intake (g)	112.75	111.6	3.2	0.873
Feed conversion ratio	4.4	4.4	0.2	0.982

C, control; TM7.5, 7.5% inclusion level of *Tenebrio molitor*.

8.3.2 Haematological and serum parameters

Dietary TM meal inclusion did not affect ($P > 0.05$) the haematological and serum parameters of the animals (Table 27).

Table 27. Effect of the dietary TM larvae meal inclusion on the haematological and serum parameters of the female free-range chickens.

	C	TM7.5	SEM	P
Erythrocytes (10^6 cell/ μ l)	2.40	2.63	0.08	0.641
Leukocytes (10^3 cell/ μ l)	9.42	9.94	0.40	0.659
H/L ratio	0.55	0.51	0.05	0.798
Albumin (g/dl)	1.04	1.31	0.08	0.174
Total protein (g/dl)	3.98	4.05	0.15	0.293
AST (U/l)	190.49	197.97	3.99	0.649
ALT (U/l)	14.49	13.99	1.53	0.411
Uric Acid (mg/dl)	4.99	3.93	0.32	0.894
Creatinine (mg/dl)	0.44	0.46	0.01	0.384
Triglycerides (mg/dl)	43.45	47.81	5.99	0.296
Cholesterol (mg/dl)	78.61	78.90	4.09	0.664
Glucose (mg/dl)	262.30	243.60	7.10	0.085
Phosphorus (mg/dl)	7.31	6.45	0.88	0.148
Magnesium (mEq/l)	0.90	1.02	0.12	0.979
Iron (μ g/dl)	54.83	61.54	9.89	0.361

C, control; TM7.5, 7.5% inclusion level of *Tenebrio molitor*; H/L: heterophils/lymphocytes; AST: aspartate-aminotransferase; ALT: alanino-aminotransferase.

8.3.3 Carcass characteristics

Dietary TM meal inclusion did not influence ($P > 0.05$) the carcass characteristics of the animals (Table 28).

Table 28. Effect of the dietary TM larvae meal inclusion on the carcass characteristics of the female free-range chickens.

	C	TM7.5	SEM	P
Chilled carcass (g)	1459.3	1544.8	26.2	0.104
Breasts (g)	347.1	370.8	10.5	0.275
Thighs (g)	479.2	502.9	10.5	0.273
Thigh muscle (g)	349.3	353.7	7.9	0.793
Thigh bone (g)	83.8	89.3	2.0	0.186
Spleen (g)	4.1	3.8	0.3	0.615
Bursa of Fabricius (g)	4.3	4.2	0.4	0.873
Liver (g)	36.7	39.4	1.2	0.278
Gizzard (g)	66.7	69.5	2.6	0.603
Abdominal fat (g)	40.6	45.1	6.7	0.752

C, control; TM7.5, 7.5% inclusion level of *Tenebrio molitor*.

8.3.4 Histopathological findings

Histopathological alterations developed for spleen, thymus, bursa of Fabricius, liver, intestine and glandular stomach and were observed in all the dietary treatments. 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. The liver showed different degrees of lymphoid tissue activation. Chronic inflammation, with lymphoid tissue activation, was observed in the intestinal segments (Figure 23). The glandular stomach showed lymphoplasmacytic inflammation, with focal to multifocal lymphoid tissue activation and different degrees of severity of epithelial squamous metaplasia (Figure 24).

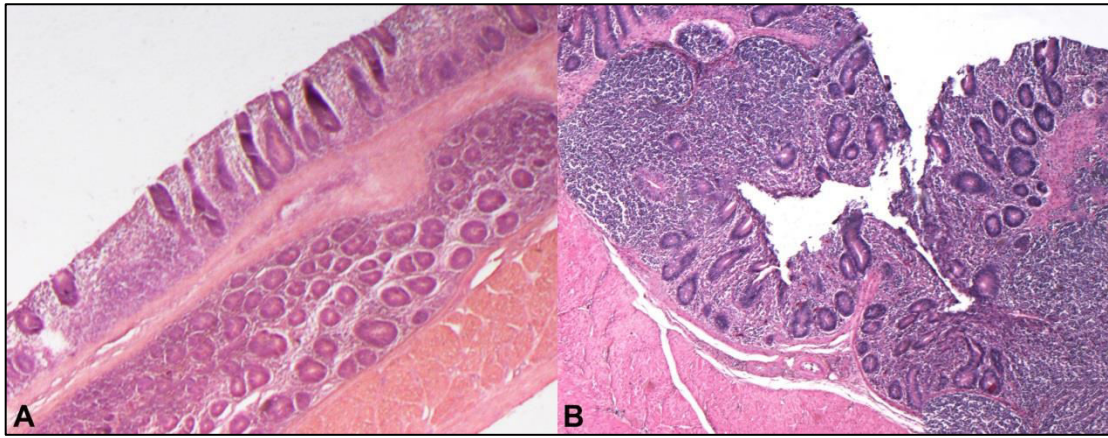


Figure 23. Histopathological findings observed in gut. **A)** A normal caecum (Haematoxylin & Eosin stain, 2.5× magnification). **B)** Mild and diffuse mucosal lymphoplasmacytic inflammation associated with severe and multifocal lymphoid tissue activation (Haematoxylin & Eosin stain, 5× magnification).

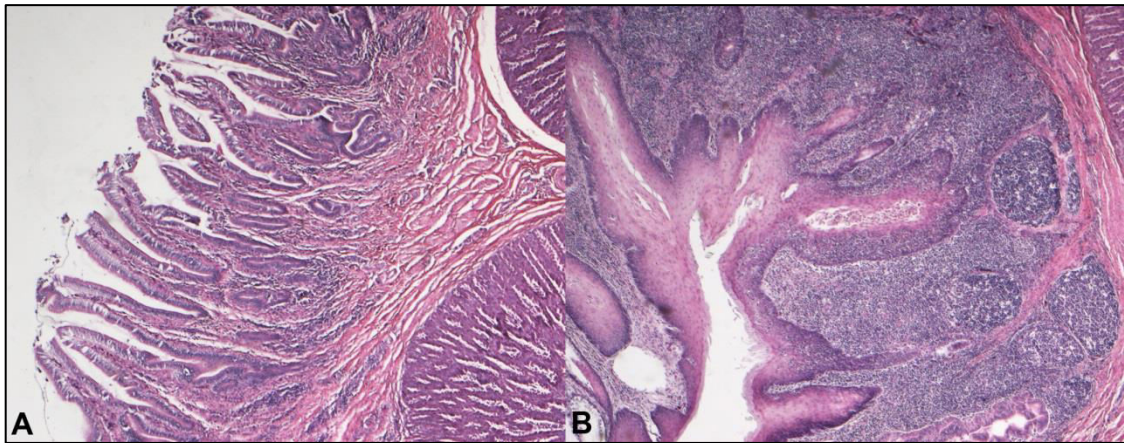


Figure 24. Histopathological findings observed in glandular stomach. **A)** A normal glandular stomach (Haematoxylin & Eosin stain, 5× magnification). **B)** Severe and diffuse mucosal lymphoplasmacytic inflammation associated with severe and multifocal lymphoid tissue activation and severe and diffuse epithelial squamous metaplasia (Haematoxylin & Eosin stain, 5× magnification).

Histopathological scores of the free-range chickens are shown in Table 29. Dietary TM meal inclusion did not significantly affect ($P > 0.05$) the severity of the histopathological findings.

Table 29. Effect of the dietary TM larvae meal inclusion on the histopathological scores of the female free-range chickens.

	C	TM7.5	SEM	P
Spleen	1.00	0.60	0.17	0.273
Thymus	0.40	0.38	0.14	0.707
Bursa of Fabricius	1.00	1.20	0.12	0.425
Liver	1.00	0.80	0.16	0.537
Duodenum	0.40	0.35	0.11	0.895
Jejunum	1.40	1.25	0.10	0.557
Ileum	1.00	0.75	0.13	0.265
Caecum	1.70	1.40	0.11	0.166
Glandular stomach	1.30	1.50	0.15	0.476
Heart	No alterations			
Kidney	No alterations			

Results are expressed as mean and pooled SEM. C = control; TM7.5 = 7.5% inclusion level of *Tenebrio molitor*.

8.3.5 Gut mucosal morphology

The morphometric data of the free-range chickens are summarized in Table 30. 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.

Table 30. Effect of the dietary TM larvae meal inclusion on the intestinal morphometric measurements of the female free-range chickens.

	C	TM7.5	SEM	P
Duodenum				
1. Villus height (mm)	2.29 ^a	2.49 ^a	0.06	0.126
2. Crypt depth (mm)	0.18	0.21 ^d	0.01	0.119
3. Villus height / crypt depth ratio (mm / mm)	13.09 ^f	12.17	0.64	0.491
Jejunum				
1. Villus height (mm)	1.94 ^b	1.93 ^b	0.08	0.945
2. Crypt depth (mm)	0.17	0.17 ^e	0.01	0.813
3. Villus height / crypt depth ratio (mm / mm)	11.29 ^{fg}	11.14	0.44	0.875
Ileum				
1. Villus height (mm)	1.60 ^c	1.72 ^b	0.07	0.405
2. Crypt depth (mm)	0.17	0.17 ^e	0.01	0.848
3. Villus height / crypt depth ratio (mm / mm)	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.

C = control; TM7.5 = 7.5% inclusion level of *Tenebrio molitor*.

8.3.6 Gut mucin composition

Mucin staining intensity in the intestinal crypts of the free-range chickens significantly depended on mucin type, gut segment and crypt fragment ($P < 0.001$). However, there was no significant influence of dietary TM meal inclusion ($P > 0.05$) on the histochemical findings (Table 31). In particular, crypts showed higher acidic sialylated mucins staining intensity ($P < 0.001$) than neutral and acidic sulfated. Furthermore, lower mucin staining intensity was found in the caecal crypts ($P < 0.001$) compared with the other gut segments and in the jejunal crypts ($P < 0.001$) compared with the duodenum and ileum, respectively. Crypt base also showed greater mucin staining intensity ($P < 0.001$) than the midsection and tip (Table 32).

There was no significant effect of dietary TM meal inclusion or mucin type ($P > 0.05$) on mucin staining intensity for the intestinal villi, whereas both gut segment and villus fragment significantly influenced ($P < 0.001$ and $P < 0.01$, respectively) the histochemical findings (Table 31). In particular, villi showed higher mucin staining intensity in the ileum ($P < 0.001$) compared with the other gut

segments and in the jejunum ($P < 0.001$) compared with the duodenum, respectively. Lower mucin staining intensity was also observed in the villus tip ($P = 0.001$) than the base (Table 32).

Table 31. Effects of diet, mucin type, gut segment and crypt-villus fragment on mucin staining intensity in the female free-range chickens.

Factor	d.f. ⁵	Chi-square	P ⁶
Crypts			
Diet ¹	1	0.765	0.382
Mucin type ²	2	34.614	< 0.001
Gut segment ³	3	145.013	< 0.001
Fragment ⁴	2	71.380	< 0.001
Villi			
Diet	1	0.002	0.965
Mucin type	2	3.032	0.220
Gut segment	2	272.016	< 0.001
Fragment	2	10.985	0.002

¹Two dietary treatments: C = control; TM7.5 = 7.5% inclusion level of *Tenebrio molitor*.

²Three types: neutral, acidic sialylated and acidic sulfated mucins.

³Four gut segments: duodenum, jejunum, ileum and caecum.

⁴Three fragments: base, midsection and tip.

⁵Degrees of freedom.

⁶Statistical significance: $P < 0.05$.

Table 32. Least square means of mucin staining intensity in the intestinal crypts and villi of the female free-range chickens in relation to diet, mucin type, gut segment and fragment.

	Predictor	Predictor factors	Mucin staining intensity ^{1,2}
Crypts	Diet	C	1.11 ± 0.03
		TM7.5	1.14 ± 0.03
	Mucin type	Neutral	1.04 ± 0.03 ^b
		Acidic sialylated	1.28 ± 0.04 ^a
		Acidic sulfated	1.06 ± 0.03 ^b
	Gut segment	Duodenum	1.38 ± 0.04 ^a
		Jejunum	1.05 ± 0.04 ^b
		Ileum	1.31 ± 0.04 ^a
		Caecum	0.83 ± 0.03 ^c
	Fragment	Base	1.36 ± 0.04 ^a
		Midsection	1.03 ± 0.03 ^b
		Tip	1.01 ± 0.03 ^b
Villi	Diet	C	1.65 ± 0.04
		TM7.5	1.65 ± 0.04
	Mucin type	Neutral	1.63 ± 0.05
		Acidic sialylated	1.72 ± 0.05
		Acidic sulfated	1.61 ± 0.05
	Gut segment	Duodenum	1.09 ± 0.04 ^c
		Jejunum	1.91 ± 0.06 ^b
		Ileum	2.16 ± 0.06 ^a
	Fragment	Base	1.78 ± 0.05 ^a
		Midsection	1.64 ± 0.05 ^{ab}
		Tip	1.55 ± 0.05 ^b

¹Data are represented as mean of counts ± SEM.

²Means with different superscript letters (a, b, c) within the same column per predictor (i.e. diet, mucin type, gut segment or fragment) differ significantly (P < 0.01).

C = control; TM7.5 = 7.5% inclusion level of *Tenebrio molitor*.

8.3.7 Cecal microbiota characterization

A total of 530.550 raw reads (2x250bp) were obtained after sequencing. After joint and quality filtering, a total of 104.081 reads passed the filters applied through QIIME, with an average value of 10.408 reads/sample. In order to avoid biases due to different sequencing depths, all samples were rarefied at 3600 reads after raw read quality filtering. The rarefaction analysis and the Good's coverage indicated a satisfactory coverage for all the samples (average Good's coverage of 84%) (Table 33). Moreover, Shannon index showed higher diversity (P < 0.05) in free-range chickens fed

with TM (average value of 7.92) compared to C diet (average value of 7.81) (Table 33). Adonis and ANOSIM statistical tests based on Weighted UniFrac distance matrix showed significant differences among dietary treatments ($P < 0.001$). These differences were demonstrated by Principal Component Analysis (PCA), which showed a clear separation of the microbiota as a function of the diet (Figure 25).

Table 33. 16S sequencing and alpha and beta diversity of the cecal microbiota of the female free-range chickens.

Diet	Description	Goods_coverage(%)	PD_whole_tree	Chao1	Observed_species	Shannon
TM7.5	FH_7.5_1	84,67	54,16	1745,94	750	7,91
TM7.5	FH_7.5_2	80,30	58,56	2254,07	892	7,81
TM7.5	FH_7.5_3	82,43	59,68	2072,66	824	8,16
TM7.5	FH_7.5_4	81,33	56,86	2360,00	855	8,09
TM7.5	FH_7.5_5	83,30	55,18	1749,86	801	7,62
C	FH_C_1	83,20	52,57	1832,98	794	7,40
C	FH_C_2	89,40	39,45	1272,22	531	6,74
C	FH_C_3	84,20	49,63	2263,96	707	7,24
C	FH_C_4	85,77	43,66	1600,64	663	7,20
C	FH_C_5	83,00	54,41	2058,95	761	7,03

Description column indicates the pool. C = control; TM7.5 = 7.5% inclusion level of *Tenebrio molitor*; FH = female hybrids.

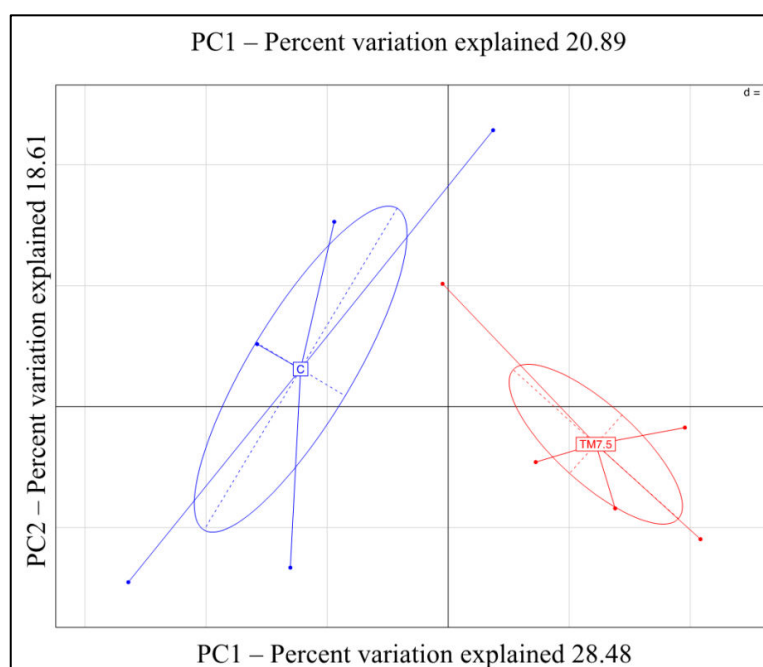


Figure 25. Bacterial community composition (weighted UniFrac beta diversity, PCA plots) in cecal samples of free-range chickens fed with control (C) and 7.5% inclusion level of *Tenebrio molitor* meal (TM7.5) diets.

Relative abundances of the main phyla and genera in the free-range chickens are summarized in Figure 26. *Bacteroidetes* represented the dominant phylum of the cecal community in both C (average relative abundance of 74.55%) and TM (58.91%) groups, outnumbering the *Firmicutes* (17.27% and 30.48%, respectively) and *Proteobacteria* (4.71 and 7.92%, respectively) phyla (Figure 26A). Within phylum *Bacteroidetes*, *Bacteroides*, unclassified members (U. m.) of *Bacteroidales* order, *Alistipes*, *Parabacteroides* and *Coproacter* reached an average relative abundance of 49.93%, 16.94%, 2.90%, 1.99% and 1.14%, respectively, in birds fed with C diet. On the other hand, TM group showed an average relative abundance of these OTUs of 37.33%, 14.19%, 2.03%, 2.37% and 1.20%, respectively. *Clostridium*, *Ruminococcus*, *Oscillospira*, *L-Ruminococcus*, *Faecalibacterium* and U. m. of *Lachnospiraceae* family were the predominant members of the *Firmicutes* phylum in both C (average relative abundances of 4.47%, 3.25%, 2.03%, 1.97%, 1.49% and 1.38%, respectively) and TM (average relative abundances of 13.4%, 4.73%, 3.12%, 1.97%, 1.85% and 1.91%, respectively) groups. Within phylum *Proteobacteria*, U. m. of *Alphaproteobacteria* class and U. m. of *Succinivibrionaceae* family were identified in both animals fed with C (average relative abundances of 1.03% and 1.14%, respectively) and TM (average relative abundances of 2.21% and 1.21%, respectively) diets (Figure 26B).

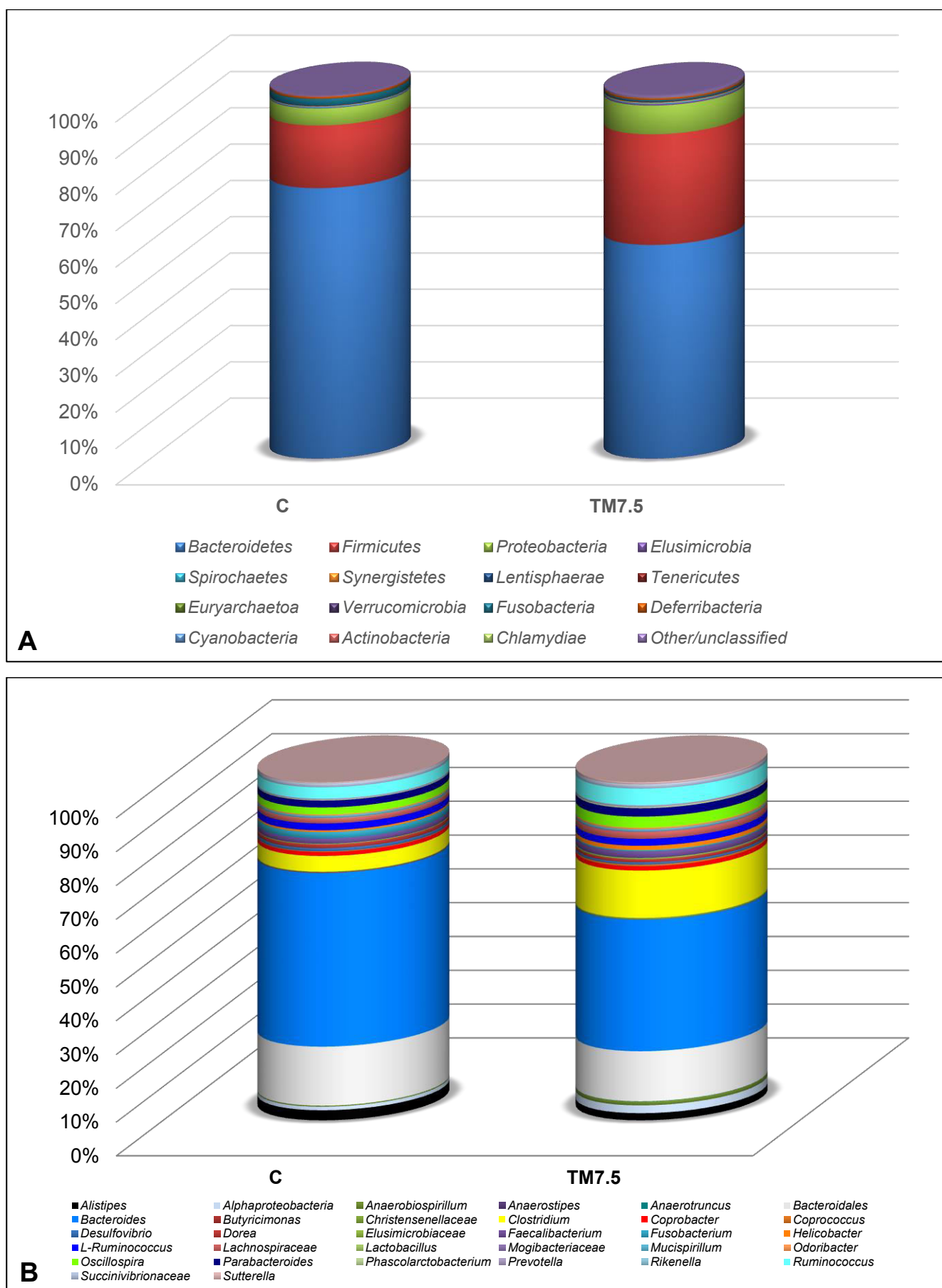


Figure 26. Relative abundance of the main bacterial phyla (A) and genera (B) in cecal samples of free-range chickens fed with control (C) and 7.5% inclusion level of *Tenebrio molitor* meal (TM7.5) diets.

Compared to the C group, the birds fed with TM displayed significant increase of the relative abundance of *Firmicutes* (FDR < 0.05, Figure 27A). On the contrary, the relative abundance of *Bacteroidetes* was lower in TM animals than C (FDR < 0.05, Figure 27B). The birds fed with TM also showed higher *Firmicutes*:*Bacteroidetes* ratios compared to the C group (FDR < 0.05, Figure 27C).

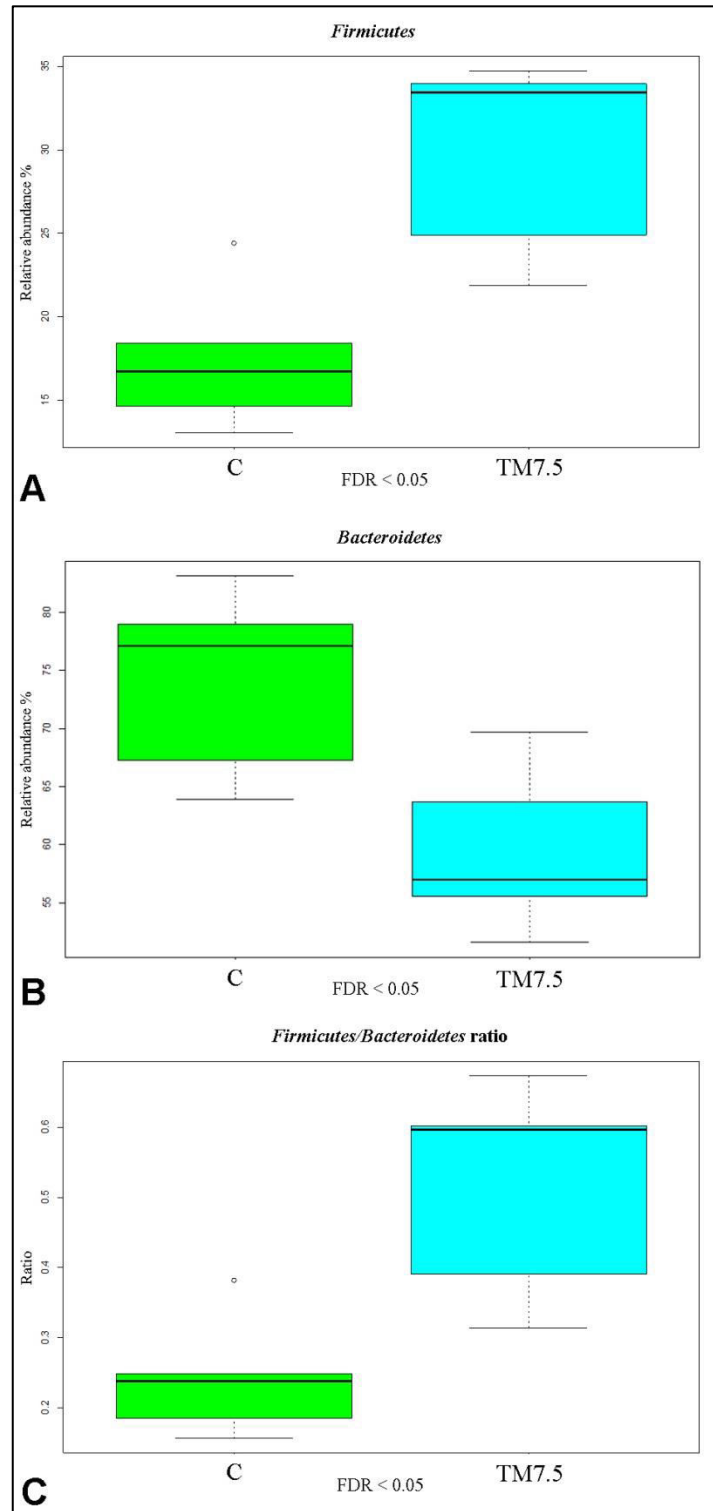


Figure 27. Boxplots of *Firmicutes* phylum (A), *Bacteroidetes* phylum (B) and *Firmicutes*:*Bacteroidetes* ratio (C) in cecal samples of free-range chickens fed with control (C) and 7.5% inclusion level of *Tenebrio molitor* meal (TM7.5) diets. Pairwise Kruskal-Wallis test, FDR < 0.05.

At genus level, the relative abundance of *Bacteroides* (Figure 28A) was lower in TM animals than C (FDR < 0.05). On the contrary, the birds fed with TM displayed significant increase of the relative abundance of *Sutterella* (Figure 28B), *Ruminococcus* (Figure 28C), *Oscillospira* (Figure 28D), *Clostridium* (Figure 28E) and *Coproccoccus* (Figure 28F) compared to the C group (FDR < 0.05).

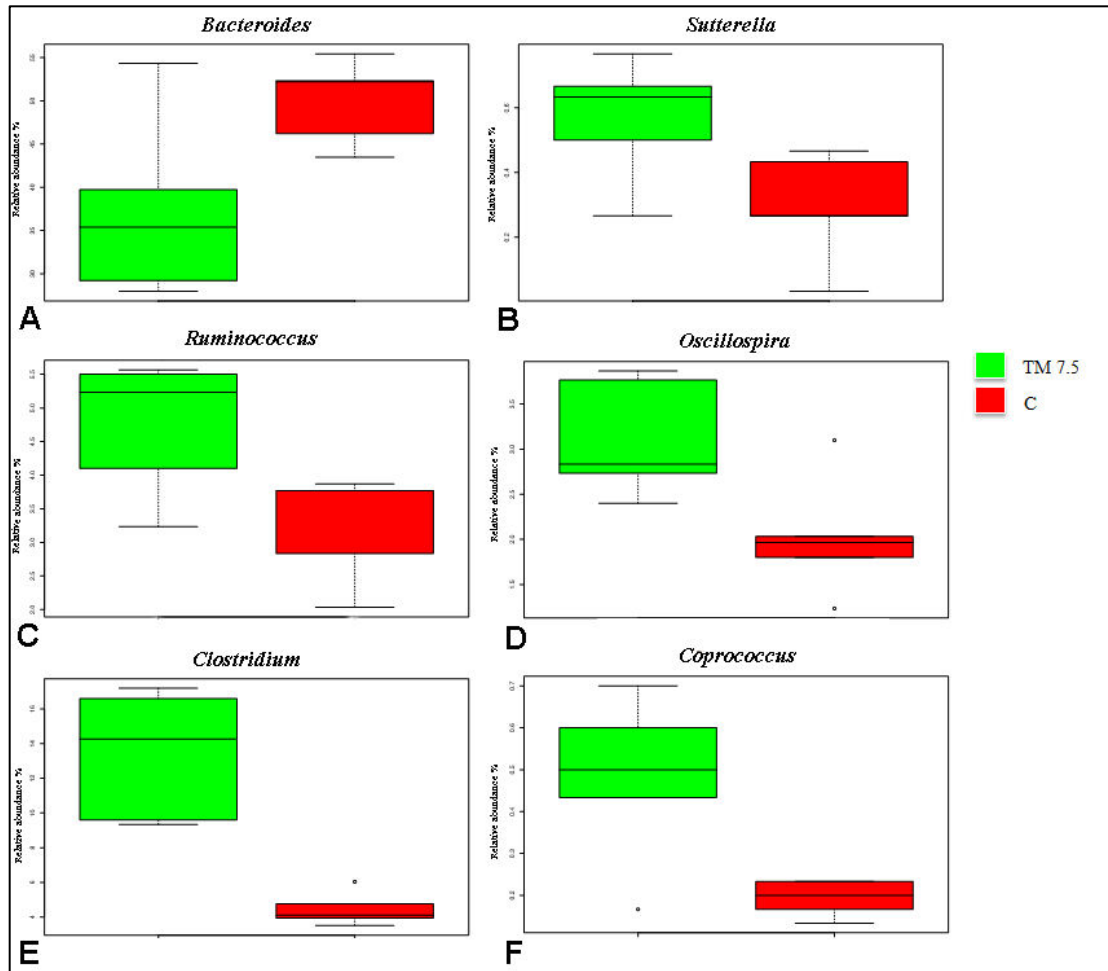


Figure 28. Boxplots of *Bacteroides* (A), *Sutterella* (B) *Ruminococcus* (C), *Oscillospira* (D), *Clostridium* (E) and *Coproccoccus* genera (F) in cecal samples of free-range chickens fed with control (C) and 7.5% inclusion level of *Tenebrio molitor* meal (TM7.5) diets. Pairwise Kruskal-Wallis test, FDR < 0.05.

8.3.8 Meat quality parameters

The pH₂₄, color and drip losses of breast and thigh of the free range chickens are reported in Table 34. All these meat quality parameters were not affected by dietary TM meal inclusion.

Table 34. Effect of the dietary TM meal inclusion level on the meat quality of breast and thigh muscles of the free-range chickens.

	C	TM7.5	SEM	P
Breast muscle				
pH	5.77	5.73	0.046	0.353
Lightness	52.14	53.03	1.045	0.405
Redness	-0.88	-0.64	0.359	0.510
Yellowness	10.42	9.75	1.424	0.645
Chroma	10.48	9.82	1.423	0.647
Hue	94.95	93.64	2.267	0.569
Drip Loss	7.40	7.99	0.817	0.478
Thigh muscle				
pH	6.28	6.39	0.088	0.241
Lightness	54.31	54.53	1.747	0.898
Redness	0.98	0.80	0.482	0.710
Yellowness	7.03	6.37	1.457	0.660
Chroma	7.23	6.59	1.406	0.653
Hue	79.19	80.54	5.788	0.819
Drip Loss	7.05	7.01	0.576	0.950

C, control; TM7.5, 7.5% inclusion level of *Tenebrio molitor*.

The proximate composition (water, ash, crude protein and ether extract) of breast and thigh meat was also not affected by dietary TM meal inclusion (Table 35).

Table 35. Effect of the dietary TM meal inclusion level on the chemical composition (% fresh matter) of breast and thigh muscles of the free-range chickens.

	C	TM7.5	SEM	P
Breast muscle				
Water	74.09	74.00	0.231	0.714
Ash	1.26	1.22	0.034	0.256
Crude protein	24.49	24.36	0.259	0.619
Ether extract	0.41	0.50	0.069	0.220
Thigh muscle				
Water	73.52	73.17	0.724	0.631
Ash	1.08	1.07	0.017	0.487
Crude protein	21.23	21.07	0.814	0.716
Ether extract	5.34	5.64	0.825	0.776

C, control; TM7.5, 7.5% inclusion level of *Tenebrio molitor*.

Table 36 shows the effects of dietary TM meal inclusion on the composition of individual and groups of FA in the breast and thigh meat of the free-range chickens. Significant differences were observed in FA profile of breast meat, while no significant differences were observed in thigh meat. The predominant FA in the breast meat of chickens fed with both C and TM7.5 diets was oleic acid (137.030 and 178.698 mg/100g FM, respectively), followed by palmitic acid (133.872 and 154.806 mg/100g FM in C and TM7.5 diet, respectively). The substitution of gluten meal with TM meal significantly affected C14:0 ($P < 0.01$), C18:3 n3 ($P < 0.01$) and C20:4 n6 ($P < 0.05$) content. Among groups of FA, higher and significant values were observed for total n-3 in TM7.5 diet samples and subsequently lower value was reported for n-6/n-3 FA ratio. A positive numerical trend was observed in Σ PUFA/ Σ saturated FA (SFA, $P = 0.090$). MUFA concentration, mainly represented by C18:1 c9, showed the highest levels in TM7.5 group despite no significant differences being observed between groups.

Table 36. Effect of the dietary TM meal inclusion level on THE fatty acid composition (g/100 g of FM) of breast and thigh muscles of the free-range chickens.

	Breast muscle				Thigh muscle			
	C	TM7.5	SEM	P	C	TM7.5	SEM	P
C12	0.306	0.262	0.046	0.356	7.867	8.590	0.741	0.342
C14	2.992	4.195	0.398	0.007	26.990	36.087	5.404	0.110
C16	133.872	154.806	18.938	0.284	1080.386	1131.260	208.149	0.810
C16:1 c9	21.238	23.930	4.036	0.513	268.888	256.457	65.996	0.853
C18	36.501	43.614	6.121	0.260	280.424	309.310	38.252	0.460
C18:1 c9	137.030	178.698	26.362	0.131	1542.272	1702.314	302.201	0.603
C18:1 c11	13.327	15.131	2.216	0.426	107.978	112.524	21.887	0.838
C18:2 n6	62.041	75.201	9.306	0.174	632.081	648.849	101.477	0.871
C18:3 n3	0.784	1.414	0.216	0.009	15.677	17.613	3.173	0.549
C18:3 n6	-	-	-	-	2.363	2.877	0.457	0.276
C20	1.818	1.944	0.297	0.675	1.403	1.813	0.388	0.304
C20:1 c11	-	-	-	-	10.364	12.380	1.868	0.295
C20:2 n6	-	-	-	-	4.115	4.791	0.737	0.371
C20:3 n6	-	-	-	-	5.037	5.399	0.728	0.625
C20:4 n6	7.724	11.812	1.890	0.044	36.965	36.001	5.726	0.868
ΣSFA	175.489	204.821	25.292	0.261	1397.070	1487.060	249.140	0.722
ΣMUFA	171.595	217.759	31.730	0.163	1929.502	2083.675	387.064	0.695
ΣPUFA	70.549	88.427	11.026	0.122	696.237	715.528	109.815	0.863
ΣPUFA/SFA	0.399	0.436	0.020	0.090	0.505	0.489	0.031	0.599
Σn3	0.784	1.414	0.216	0.009	15.677	17.613	3.173	0.549
Σn6	69.765	87.014	10.855	0.129	680.560	697.916	106.789	0.873
Σn6/n3	91.994	64.731	6.618	0.001	44.602	41.503	2.486	0.229
AI	0.627	0.567	0.028	0.042	0.455	0.463	0.020	0.688
TI	1.452	1.304	0.052	0.011	0.996	1.004	0.040	0.840
TFA (g/100 g of FM)	417.633	511.006	67.167	0.181	4022.808	4284.354	734.557	0.726

C, control; TM7.5, 7.5% inclusion level of *Tenebrio molitor*;

SFA, saturated fatty acids = (C12+C14+C15+C16+C18+C20+C22); MUFA, monounsaturated fatty acids = (C14:1 c9+C16:1 c9+C17:1 c9+C18:1 c9+C18:1 c9+C18:1 c11+C20:1 c11); PUFA, polyunsaturated fatty acids = (C18:2 n6+C18: 3 n3+C18:3 n6+C20:2 n6+C20:3 n6+C20:4 n6); AI, atherogenic index; TI, thrombogenic index; TFA, total fatty acids.

8.4 EXPERIMENT 4

Part of the results related to the experimental trial 4 have recently been submitted to Journal of Animal Science and Biotechnology.

8.4.1 Growth performance

During the whole experimental period, birds showed all vitalities (no signs of illness were observed and the mortality rate was zero in all groups). Table 37 summarizes the growth performance of the broiler chickens. The initial body weight of chicks did not differ ($P > 0.05$) among the different dietary treatments. At 10, 24 and 35 days of age, the LW showed linear and quadratic response to increasing HI meal up to 10% ($P < 0.05$). However, there was minimal change in LW with increasing amounts of supplementation beyond 10%. Similar results were observed for ADG during the study. In the periods from 1 to 10 and from 10 to 24 days of age, the ADG increased up to the moderate level of supplementation and then declined at the highest level. In contrast, linear decrease was elicited by HI meal as the level of supplementation increased from 0 to 15% during the finisher period. In the period from 1 to 10 days of age, the DFI increased linearly and quadratically with increasing HI meal levels ($P < 0.05$) and the linear and quadratic responses increased to a maximum corresponding to the inclusion of 10% of HI meal. On the contrary, the DFI showed no differences ($P > 0.05$) in the periods from 10 to 24 and 24 to 35 days of age. In the period from 1 to 10 days of age, the FCR was similar among the dietary treatments ($P > 0.05$). Linear and quadratic responses were observed between the groups in the periods from 10 to 24 days of age and from 1 to 35 days of age, with a maximum corresponding to the inclusion of 150 g/kg of HI meal ($P < 0.001$). Differently, the FCR showed linear response to increasing HI meal levels in the period from 24 to 35 ($P < 0.05$), with a maximum corresponding to the inclusion of 150 g/kg of HI meal.

Table 37. Effect of the dietary HI larvae meal inclusion level on the growth performances of the male broiler chickens.

Variable ²	Age	Dietary treatments ¹				SEM	P ³	
		C	HI5	HI10	HI15		Linear	Quadratic
LW (g)	1 d	40.23	40.20	40.33	40.23	0.020	0.443	0.363
	10 d	262.19	259.22	285.23	267.89	2.349	0.005	0.030
	24 d	1194.91	1199.45	1227.89	1095.23	10.157	0.000	0.000
	35 d	2269.25	2264.11	2278.95	2072.15	18.173	0.000	0.000
DWG (g)	1-10 d	22.20	21.90	24.49	22.77	0.234	0.005	0.031
	10-24 d	66.62	67.16	67.33	59.10	0.716	0.000	0.000
	24-35d	97.67	96.79	95.55	88.81	1.187	0.007	0.179
DFI (g)	1-10 d	25.89	25.64	28.52	26.54	0.279	0.012	0.040
	10-24 d	97.50	96.69	98.70	95.23	0.572	0.340	0.238
	24-35 d	176.42	174.62	174.27	171.73	0.988	0.115	0.852
FCR (g/g)	1-10 d	1.17	1.17	1.16	1.17	0.005	0.866	0.900
	10-24 d	1.46	1.44	1.47	1.61	0.016	0.000	0.001
	24-35 d	1.81	1.81	1.83	1.93	0.018	0.011	0.136
	1-35 d	1.60	1.59	1.60	1.72	0.012	0.000	0.000

¹Four dietary treatments: C = control; HI5 = 5% inclusion level of *Hermetia illucens*; HI10 = 10% inclusion level of *Hermetia illucens*; HI15 = 15% inclusion level of *Hermetia illucens*.

²LW, live weight; DWG, daily weight gain; DFI, daily feed intake; FCR, feed conversion ratio.

³Statistical significance: P < 0.05.

8.4.2 Haematological and serum parameters

Haematological and serum parameters of the broiler chickens are summarized in Table 38. No significant effects related to HI meal utilization were observed ($P > 0.05$) except for phosphorus concentration, which showed a linear and quadratic response to increasing HI meal levels ($P < 0.001$), with a maximum corresponding to the inclusion of 100 g/kg of HI meal.

Table 38. Effect of the dietary HI larvae meal inclusion level on the haematological and serum parameters of the male broiler chickens

Variable ²	Dietary treatments ¹				SEM	P ³	
	C	HI5	HI10	HI15		Linear	Quadratic
Erythrocyte (10 ⁶ cell/ μ l)	4.18	3.94	3.93	3.98	0.046	0.132	0.122
Leukocyte (10 ³ cell/ μ l)	13.70	13.55	13.77	14.32	0.246	0.344	0.492
H/L ratio	0.66	0.68	0.65	0.69	0.028	0.838	0.824
Urea (g/dl)	34.88	39.53	42.59	36.30	1.980	0.712	0.177
Uric Acid (mg/dl)	2.89	2.67	2.46	2.77	0.180	0.750	0.477
AST (UI/l)	379.57	367.52	366.33	358.78	13.508	0.437	0.904
Creatinine (mg/dl)	0.29	0.27	0.27	0.28	0.006	0.546	0.542
Triglycerides (mg/dl)	105.25	94.70	97.82	84.32	3.541	0.057	0.826
Cholesterol (mg/dl)	81.34	81.07	85.73	82.64	2.044	0.660	0.734
Calcium (mg/dl)	8.77	7.56	8.43	8.42	0.209	0.947	0.155
Phosphorus (mg/dl)	3.91	5.90	6.02	5.34	0.172	0.000	0.000
Magnesium (mEq/l)	1.12	1.04	0.96	0.92	0.055	0.186	0.837
Iron (μ g/dl)	59.89	61.12	66.03	61.72	3.866	0.780	0.727

¹Four dietary treatments: C = control; HI5 = 5% inclusion level of *Hermetia illucens*; HI10 = 10% inclusion level of *Hermetia illucens*; HI15 = 15% inclusion level of *Hermetia illucens*.

²AST, aspartate amino transferase.

³Statistical significance: P < 0.05.

8.4.3 Carcass characteristics

The carcass characteristics of the broiler chickens are presented in Table 39. Linear and quadratic response was observed to increasing HI meal levels in LW, carcass weight, breast and thigh muscles weights ($P < 0.001$), with a maximum corresponding to the inclusion of 100 g/kg of HI meal. The breast weight yield increased quadratically with increasing HI meal levels ($P < 0.05$) and the quadratic response increased to a maximum corresponding to the inclusion of 100 g/kg of HI meal. The heart weight linearly decreased with increasing HI meal levels ($P < 0.05$) and the linear response decreased to a minimum corresponding to the inclusion of 150 g/kg of HI meal. The abdominal fat weight yield showed linear response to increasing HI meal levels ($P < 0.05$), with a maximum corresponding to the inclusion of 150 g/kg of HI meal. On the contrary, no significant effects related to HI meal utilization were observed for the other carcass traits ($P > 0.05$).

Table 39. Effect of dietary HI larvae meal inclusion level on the carcass characteristics of the male broiler chickens.

Variable ²	Dietary treatments ¹				SEM	P ³	
	C	HI 5	HI 10	HI15		Linear	Quadratic
Live weight (LW) (g)	2260.56	2259.44	2266.88	2070.13	15.689	<0.001	0.000
Carcass weight (g)	1594.84	1601.01	1607.84	1469.65	11.734	<0.001	0.000
Carcass weight (% LW)	70.55	70.86	70.92	71.00	0.199	0.453	0.785
Breast (g)	326.92	331.63	336.36	280.80	5.020	0.000	0.000
Breast (% LW)	14.46	14.68	14.84	13.57	0.165	0.067	0.018
Thigh (g)	422.15	420.11	425.81	381.95	4.108	0.000	0.001
Thigh (% LW)	18.67	18.59	18.79	18.45	0.121	0.676	0.611
Spleen (g)	2.60	2.37	2.19	2.23	0.102	0.174	0.513
Spleen (% LW)	0.11	0.10	0.09	0.11	0.005	0.486	0.262
Liver (g)	49.10	48.21	47.21	46.58	1.149	0.430	0.957
Liver (% LW)	2.17	2.13	2.08	2.25	0.055	0.707	0.368
Heart (g)	14.39	13.50	12.76	11.84	0.336	0.005	0.984
Heart (% LW)	0.64	0.60	0.56	0.57	0.014	0.078	0.400
Bursa of Fabricius (g)	6.52	5.88	5.84	5.48	0.258	0.191	0.794
Bursa of Fabricius (% LW)	0.29	0.26	0.26	0.27	0.012	0.532	0.476
Abdominal fat (g)	27.37	26.52	32.45	29.95	1.142	0.183	0.717
Abdominal fat (% LW)	1.21	1.17	1.43	1.44	0.053	0.042	0.813

¹Four dietary treatments: C = control; HI5 = 5% inclusion level of *Hermetia illucens*; HI10 = 10% inclusion level of *Hermetia illucens*; HI15 = 15% inclusion level of *Hermetia illucens*.

²LW, live weight.

³Statistical significance: P < 0.05.

8.4.4 Histopathological findings

Histopathological alterations developed for spleen, thymus, bursa of Fabricius and liver and were observed in all the dietary treatments. 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. Finally, the liver showed different degrees of lymphoid tissue activation. Histopathological scores of the broiler chickens are shown in Table 40. Dietary HI meal inclusion did not significantly affect ($P > 0.05$) the severity of the histopathological findings.

Table 40. Effects of dietary HI larvae meal inclusion on the histopathological scores of the male broiler chickens.

	C	HI5	HI10	HI15	SEM	P
Spleen	1.08	1.17	1.33	0.83	0.09	0.259
Thymus	0.17	0.00	0.25	0.33	0.06	0.312
Bursa of Fabricius	1.08	0.75	0.75	0.75	0.09	0.430
Liver	0.92	0.50	0.92	0.33	0.11	0.174
Intestine	No alterations					
Glandular stomach	No alterations					
Heart	No alterations					
Kidney	No alterations					

Results are expressed as mean and pooled SEM. C = control; HI5 = 5% inclusion level of *Hermetia illucens*; HI10 = 10% inclusion level of *Hermetia illucens*; HI15 = 15% inclusion level of *Hermetia illucens*.

8.4.5 Gut mucosal morphology

The effects of the diet, intestinal segment and interaction between diet and intestinal segment on the intestinal morphometric indices are summarized in Table 41 and 42. Vh and Cd depended on diet ($P < 0.05$) and intestinal segment ($P < 0.001$), while, there was no influence of interaction between diet and intestinal segment ($P > 0.05$) on the morphometric indices (Table 41). In particular, HI15 birds showed lower Vh than the other groups ($P < 0.05$) and higher Cd than C and HI5 ($P < 0.05$). Furthermore, higher Vh and Cd were found in the duodenum compared with the other gut segments ($P < 0.001$ and $P < 0.01$, respectively) (Table 42). Diet ($P < 0.01$), intestinal segment ($P < 0.001$) and interaction between diet and intestinal segment ($P < 0.05$) all influenced ($P < 0.05$) the Vh/Cd (Table 41). In particular, HI15 birds showed lower Vh/Cd than the other groups ($P < 0.01$). Furthermore, higher Vh/Cd was found in the duodenum compared with the other gut segments ($P < 0.01$) (Table 42).

Table 41. Effects of diet, intestinal segment and interaction between diet and intestinal segment on the intestinal morphometric indices of the male broiler chickens.

Index	Fixed effect	d.f. ³	F	P ⁴
Vh (mm)	Diet ¹	3	2.893	0.038
	Intestinal segment ²	2	53.448	< 0.001
	Diet × Intestinal segment	6	0.922	0.482
Cd (mm)	Diet	3	3.223	0.040
	Intestinal segment	2	10.130	< 0.001
	Diet × Intestinal segment	6	1.988	0.072
Vh/Cd (mm/mm)	Diet	3	5.024	0.002
	Intestinal segment	2	17.858	< 0.001
	Diet × Intestinal segment	6	2.274	0.040

¹Four dietary treatments: C = control; HI5 = 5% inclusion level of *Hermetia illucens*; HI10 = 10% inclusion level of *Hermetia illucens*; HI15 = 15% inclusion level of *Hermetia illucens*.

²Three intestinal segments: duodenum, jejunum and ileum.

³Degrees of freedom.

⁴Statistical significance: P < 0.05.

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

Table 42. Least square means of the intestinal morphometric indices in the male broiler chickens in relation to diet and intestinal segment.

Index	Fixed effect	Effect levels	Least square mean ¹	SEM
Vh (mm)	Diet ²	C	1.66 ^a	0.05
		HI5	1.64 ^a	
		HI10	1.70 ^a	
		HI15	1.49 ^b	
	Intestinal segment ³	DU	1.99 ^a	0.05
		JE	1.57 ^b	
		IL	1.30 ^c	
Cd (mm)	Diet	C	0.16 ^a	0.01
		HI5	0.17 ^a	
		HI10	0.17 ^{ab}	
		HI15	0.19 ^b	
	Intestinal segment	DU	0.18 ^a	0.00
		JE	0.16 ^{ab}	
		IL	0.15 ^b	
Vh/Cd (mm/mm)	Diet	C	10.79 ^a	0.41
		HI5	10.83 ^a	
		HI10	10.45 ^a	
		HI15	8.89 ^b	
	Intestinal segment	DU	11.71 ^a	0.36
		JE	10.14 ^b	
		IL	8.79 ^c	

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

²C = control; HI5 = 5% inclusion level of *Hermetia illucens*; HI10 = 10% inclusion level of *Hermetia illucens*; HI15 = 15% inclusion level of *Hermetia illucens*.

³DU = duodenum; JE = jejunum; IL = ileum.

8.4.6 Gut mucin composition

Mucin staining intensity in the intestinal crypts of broiler chickens significantly depended on mucin type ($P < 0.001$), gut segment ($P < 0.001$) and crypt fragment ($P < 0.001$), while there was no significant influence of dietary HI meal inclusion ($P > 0.05$) on the histochemical findings (Table 43). In particular, crypts showed higher neutral and acidic sialylated mucins staining intensity ($P < 0.001$) than acidic sulfated. Furthermore, higher mucin staining intensity was found in the ileal crypts ($P < 0.001$) compared with the other gut segments. Crypt base also showed greater mucin staining

intensity ($P < 0.001$) than the midsection and tip, with a significant decrease ($P < 0.001$) also observed from the midsection to the tip (Table 44).

Dietary HI meal inclusion ($P < 0.001$) and gut segment ($P < 0.001$) significantly influenced the mucin staining intensity in the intestinal villi, whereas a statistical trend was observed for mucin type ($P = 0.10$). On the contrary, there was no significant effect of villus fragment ($P > 0.05$) on the histochemical findings (Table 43). In particular, villi of HI10 and HI15 animals showed lower mucin staining intensity ($P < 0.01$) compared with C and HI5. Furthermore, greater acidic sialylated mucin staining intensity ($P < 0.05$) than acidic sulfated was observed. Ileal villi also showed higher mucin staining intensity ($P < 0.001$) than the other gut segments, with a significant increase ($P < 0.001$) also observed from the duodenum to the jejunum (Table 44).

Table 43. Effects of diet, mucin type, gut segment and crypt-villus fragment on mucin staining intensity in the male broiler chickens.

Factor	d.f. ⁵	Chi-square	P ⁶
Crypts			
Diet ¹	3	3.751	0.290
Mucin type ²	2	22.566	< 0.001
Gut segment ³	3	63.140	< 0.001
Fragment ⁴	2	247.461	< 0.001
Villi			
Diet	3	25.497	< 0.001
Mucin type	2	4.510	0.10
Gut segment	2	571.512	< 0.001
Fragment	2	1.488	0.475

¹Four dietary treatments: C = control; HI5 = 5% inclusion level of *Hermetia illucens*; HI10 = 10% inclusion level of *Hermetia illucens*; HI15 = 15% inclusion level of *Hermetia illucens*.

²Three types: neutral, acidic sialylated and acidic sulfated mucins.

³Four gut segments: duodenum, jejunum, ileum and caecum.

⁴Three fragments: base, midsection and tip.

⁵Degrees of freedom.

⁶Statistical significance: $P < 0.05$.

Table 44. Least square means of mucin staining intensity in the intestinal crypts of the male broiler chickens in relation to diet, mucin type, gut segment and crypt fragment.

	Predictor	Predictor factors	Mucin staining intensity ^{1,2}
Crypts	Diet	C	1.23 ± 0.03
		HI5	1.27 ± 0.03
		HI10	1.27 ± 0.03
		HI15	1.30 ± 0.03
	Mucin type	Neutral	1.32 ± 0.03 ^A
		Acidic sialylated	1.31 ± 0.02 ^A
		Acidic sulfated	1.18 ± 0.02 ^B
	Gut segment	Duodenum	1.26 ± 0.03 ^B
		Jejunum	1.20 ± 0.03 ^{BC}
		Ileum	1.45 ± 0.03 ^A
		Caecum	1.19 ± 0.03 ^C
	Fragment	Base	1.59 ± 0.03 ^A
		Midsection	1.17 ± 0.02 ^B
		Tip	1.10 ± 0.02 ^C
Villi	Diet	C	1.90 ± 0.04 ^A
		HI5	1.87 ± 0.04 ^A
		HI10	1.72 ± 0.04 ^B
		HI15	1.66 ± 0.04 ^B
	Mucin type	Neutral	1.79 ± 0.04 ^{ab}
		Acidic sialylated	1.84 ± 0.04 ^a
		Acidic sulfated	1.74 ± 0.03 ^b
	Gut segment	Duodenum	1.23 ± 0.03 ^C
		Jejunum	1.95 ± 0.04 ^B
		Ileum	2.37 ± 0.04 ^A
	Fragment	Base	1.75 ± 0.03
		Midsection	1.81 ± 0.04
		Tip	1.80 ± 0.04

¹Data are represented as mean of counts ± SEM.

²Means with different superscript letters (a, b or A, B, C) within the same column per predictor (i.e. diet, mucin type, gut segment or fragment) differ significantly ($P < 0.05$ or $P < 0.01$, respectively).

C = control; HI5 = 5% inclusion level of *Hermetia illucens*; HI10 = 10% inclusion level of *Hermetia illucens*; HI15 = 15% inclusion level of *Hermetia illucens*.

8.4.7 Cecal microbiota characterization

A total of 1.716.304 raw reads (2x250bp) were obtained after sequencing. After joint and quality filtering, a total of 1.602.517 reads passed the filters applied through QIIME, with an average value of 69.674 reads/sample and a median sequence length of 441 bp. The rarefaction analysis and the Good's coverage indicated a satisfactory coverage for all the samples (average Good's coverage of 94%) (Table 45). Moreover, Shannon index showed lower diversity ($P < 0.05$) in broiler chickens fed with HI15 (average value of 6.49) when compared to the other diets (C, average value of 7.25; HI5, average value of 6.48; HI10, average value of 7.36) (Table 45). Adonis and ANOSIM statistical tests based on Weighted UniFrac distance matrix showed significant differences among dietary treatments ($P < 0.001$). These differences were demonstrated by Principal Component Analysis (PCA), which showed a separation of the microbiota as a function of the diet. The distinction was particularly evident for broiler chickens fed with HI5 (Figure 29).

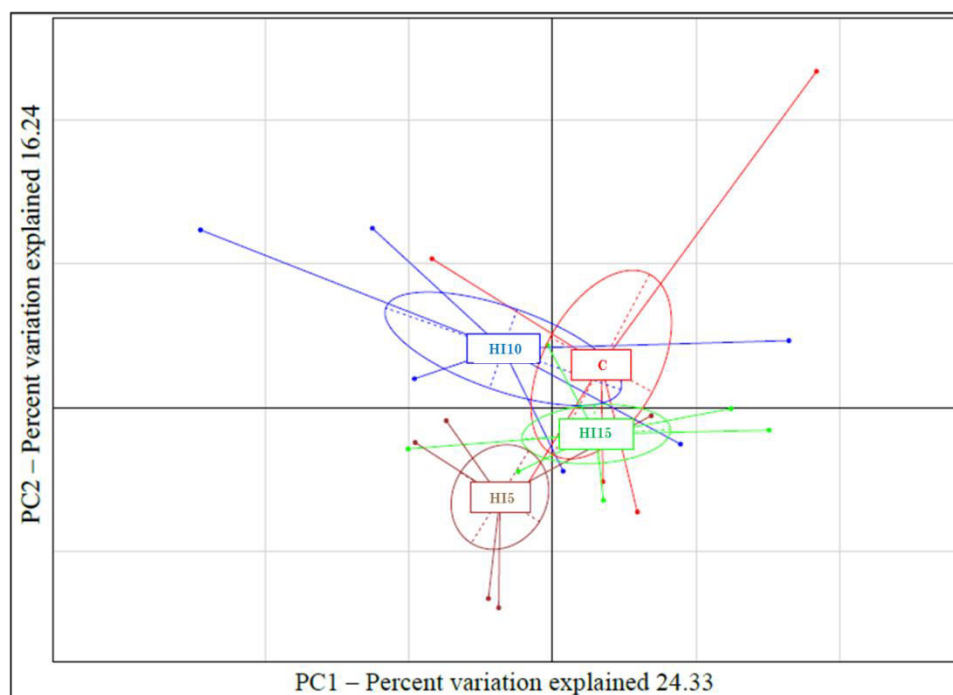


Figure 27. Bacterial community composition (weighted UniFrac beta diversity, PCA plots) in cecal samples of male broiler chickens fed with control (C), 5% (HI5), 10% (HI10) and 15% (HI15) inclusion level of *Hermetia illucens* meal diets.

Table 45. 16S sequencing and alpha and beta diversity of the cecal microbiota of the male broiler chickens.

Diet	Description	Goods_coverage(%)	PD_whole_tree	Chao1	Observed_species	Shannon
C	M_C_1	94,05	79,21	2626,26	1130,00	7,30
C	M_C_2	95,77	60,18	1904,44	844,00	6,91
C	M_C_3	95,81	62,15	1910,31	901,00	7,56
C	M_C_4	90,23	104,58	3936,48	1629,00	7,40
C	M_C_5	92,23	90,52	2783,76	1360,00	6,89
C	M_C_6	93,78	79,08	2920,41	1104,00	7,40
HI5	M_5_1	96,93	46,42	1176,44	628,00	4,62
HI5	M_5_2	94,80	68,87	2148,35	987,00	7,00
HI5	M_5_3	93,62	81,19	2845,81	1096,00	6,86
HI5	M_5_4	96,45	57,74	1508,38	774,00	7,01
HI5	M_5_5	89,46	115,42	4411,57	1639,00	6,93
HI10	M_10_1	94,53	78,48	2147,67	1054,00	7,39
HI10	M_10_2	94,30	76,76	2444,99	1027,00	6,98
HI10	M_10_3	95,05	66,19	2088,88	947,00	7,23
HI10	M_10_4	91,30	105,75	3129,29	1528,00	7,78
HI10	M_10_5	95,29	65,77	2024,55	939,00	7,56
HI10	M_10_6	94,74	71,58	2074,78	1018,00	7,23
HI15	M_15_1	95,30	60,90	1937,47	884,00	6,51
HI15	M_15_2	95,84	62,12	1689,00	811,00	6,02
HI15	M_15_3	97,78	38,83	915,55	544,00	5,27
HI15	M_15_4	90,65	104,67	3833,50	1507,00	7,34
HI15	M_15_5	94,03	78,20	2504,45	1049,00	6,85
HI15	M_15_6	95,09	70,70	2082,16	943,00	6,94

Description column indicates the pool. C = control; HI5 = 5% inclusion level of *Hermetia illucens*; HI10 = 10% inclusion level of *Hermetia illucens*; HI15 = 15% inclusion level of *Hermetia illucens*; M = male broilers.

Relative abundances of the main phyla and genera in the broiler chickens are summarized in Figure 30. *Firmicutes* represented the dominant phylum of the cecal community in all groups (C: 85.97%, HI5: 86.22%, HI10: 87.82% and HI15: 74.55%), outnumbering the *Proteobacteria* (C: 4.18%, HI5: 8.66%, HI10: 2.83% and HI15: 12.28%, respectively) and *Bacteroidetes* (C: 3.21%, HI5: 1.60%, HI10: 4.95% and HI15: 9.41%, respectively) phyla (Figure 30A). Unclassified members (U. m.) of *Clostridiales* order, U. m. of Ruminococcaceae family, *Faecalibacterium*, *Oscillospira*, U. m. of *Lachnospiraceae* family, *Ruminococcus*, *L-Ruminococcus*, U. m. of *Erysipelotrichaceae* family and *Lactobacillus* were the predominant members of the *Firmicutes* phylum in C (27.94%, 12.20%, 13.12%, 6.59%, 6.06%, 5.01%, 3.21%, 1.33% and 1.28%, respectively), HI5 (18.44%, 11.58%, 18.32%, 6.97%, 5.07%, 5.22%, 6.43%, 1.73% and 2.52%, respectively), HI10 (25.40%, 10.76%, 5.55%, 7.96%, 6.00%, 6.59%, 5.50%, 3.12% and 3.54%, respectively) and HI15 (20.62%, 11.65%, 3.04%, 6.73%, 4.94%, 4.49%, 5.18%, 5.75% and 1.56%, respectively) groups. Within phylum *Proteobacteria*, *Helicobacter* genus reached an average relative abundance of 3.05%, 7.19%, 2.31% and 11.35% in C, HI5, HI10 and HI15 groups, respectively. *Bacteroides* genus was the predominant member of *Bacteroidetes* phylum in C (average relative abundance of 3.21%), HI5 (average relative abundance of 1.59%), HI10 (average relative abundance of 3.59%) and HI15 (average relative abundance of 9.41%) groups, respectively (Figure 30B).

Comparing the relative abundance of the main OTUs among the dietary treatments, a microbiota signature was observed for each diet. In particular, C samples were characterized by the presence of *Lachnospiraceae* (6% of the relative abundance, FDR < 0.05), while *L-Ruminococcus*, *Faecalibacterium*, *Blautia* and *Clostridium* were found to be characteristic of HI5 diet (7%, 17%, 2% and 1%, respectively, FDR < 0.05). Broiler chickens fed with HI10 were characterized (FDR < 0.05) by *Lactobacillus* (4%) and *Ruminococcus* (3%), whereas *Bacteroides* (10%), *Roseburia* (1%) and *Helicobacter* (12%) were characteristic for HI15 diet (FDR < 0.05).

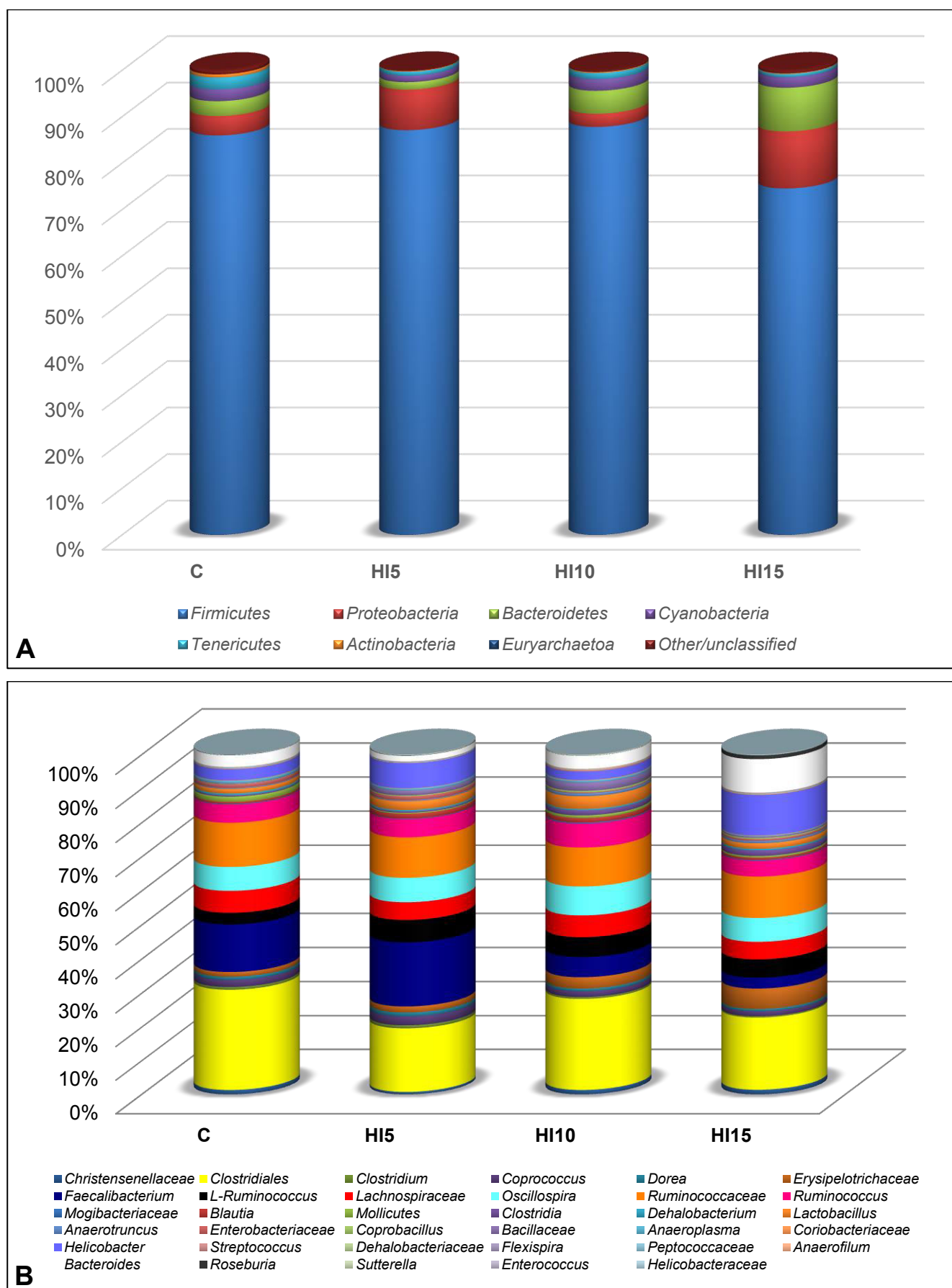


Figure 30. Relative abundance of the main bacterial phyla (A) and genera (B) in cecal samples of male broiler chickens fed with control (C), 5% (HI5), 10% (HI10) and 15% (HI15) inclusion level of *Hermetia illucens* meal diets.

Compared to the C group, the birds fed with TM displayed unaffected relative abundances of *Firmicutes* ($P > 0.05$, Figure 31A) and *Bacteroidetes* ($P > 0.05$, Figure 31B). On the contrary, the relative abundance of *Proteobacteria* was higher in HI15 animals than HI10 ($P < 0.05$, Figure 31C). The birds feed with HI also showed unaffected *Firmicutes*:*Bacteroidetes* ratios compared to the C group ($P > 0.05$, Figure 31D).

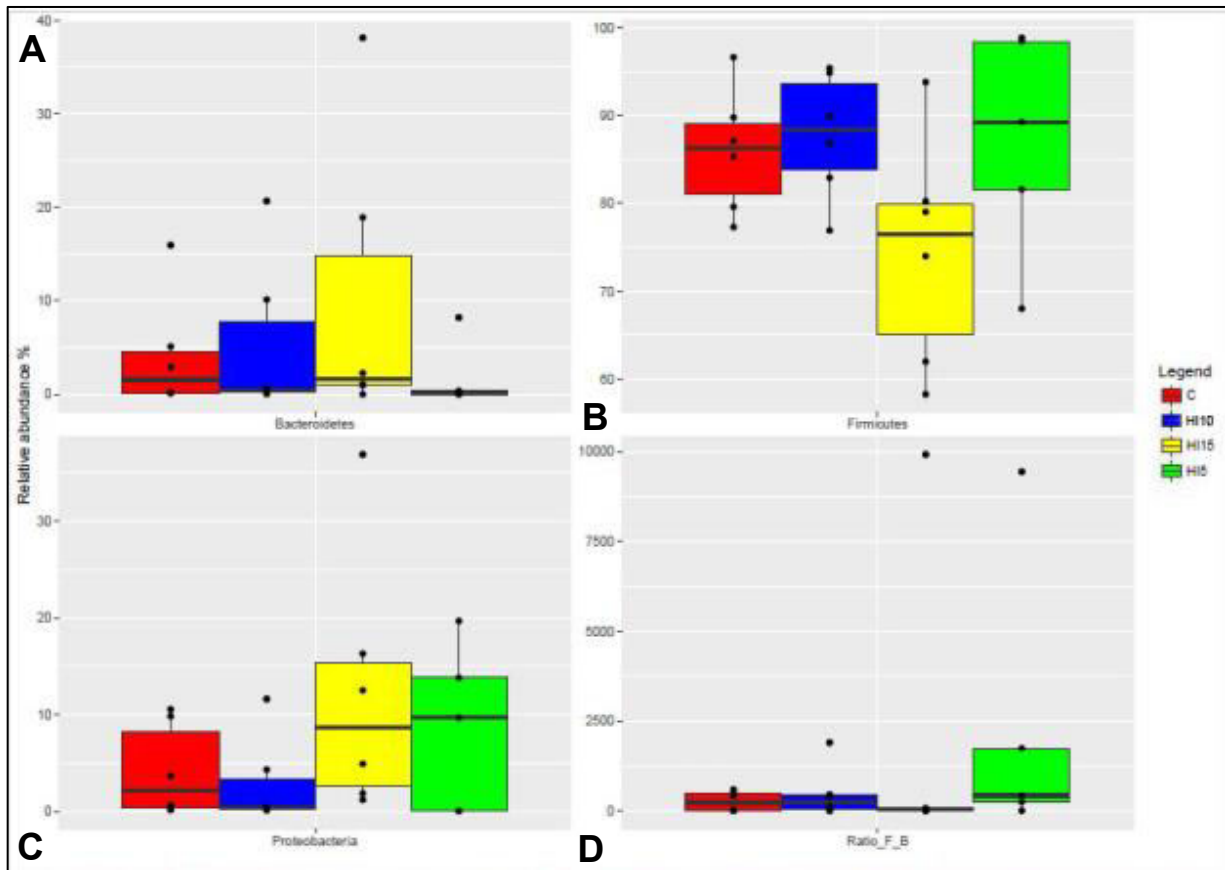


Figure 31. Boxplots of *Firmicutes* phylum (A), *Bacteroidetes* phylum (B), *Proteobacteria* phylum (C) and *Firmicutes*:*Bacteroidetes* ratio (D) in cecal samples of male broiler chickens fed with control (C), 5% (HI5), 10% (HI10) and 15% (HI15) inclusion level of *Hermetia illucens* meal diets. Pairwise Kruskal-Wallis test, $P < 0.05$.

8.4.8 Meat quality parameters

The technological properties of the breast meat are summarized in Table 46. No significant differences were observed for the technological traits ($P > 0.05$), with the exception of the redness (a^*) and yellowness (b^*) color traits. The observed redness showed a linear response to increasing HI meal levels ($P < 0.05$), with a maximum observed for the HI15 group. As far as the chemical composition is concerned, the moisture content decreased linearly with increasing HI meal levels ($P < 0.05$), with a minimum observed for the inclusion of 15% of HI meal. A linear response was observed for the protein content, with a maximum identified for the HI15 group. On the other hand, the lipid content showed a quadratic response to increasing HI meal levels, with a maximum observed for the inclusion of 10% of HI meal ($P < 0.05$). No significant effects related to HI meal utilization were observed for the ash ($P > 0.05$).

Table 46. Effect of the dietary HI larvae meal inclusion on the meat quality and chemical composition of breast muscle of the male broiler chickens.

Variable ²	Dietary treatments ¹				SEM	P ³	
	C	HI5	HI10	HI15		Linear	Quadratic
pH _u	6.03	5.99	6.04	5.98	0.052	0.650	0.777
Color							
Lightness (L*)	55.08	55.46	54.29	53.42	0.379	0.074	0.400
Redness (a*)	2.72	3.19	2.87	3.71	0.140	0.030	0.469
Yellowness (b*)	11.80	9.68	8.19	7.58	0.388	<0.001	0.174
Drip loss (%)	1.25	1.36	1.26	1.39	0.061	0.211	0.928
Cooking loss (%)	20.11	20.49	19.31	19.71	0.360	0.480	0.990
AK shear force (kg/g)	1.86	2.24	2.14	2.06	0.063	0.375	0.074
<i>Proximate composition</i>							
Moisture (%)	76.14	75.52	75.50	75.24	0.126	0.016	0.460
Protein (%)	22.37	22.28	22.42	23.09	0.124	0.035	0.118
Lipid (%)	1.56	1.77	2.19	1.75	0.072	0.098	0.017
Ash (%)	1.24	1.33	1.24	1.31	0.019	0.427	0.718

¹Four dietary treatments: C = control; HI5 = 5% inclusion level of *Hermetia illucens*; HI10 = 10% inclusion level of *Hermetia illucens*; HI15 = 15% inclusion level of *Hermetia illucens*.

²L*, lightness; a*, redness; b*, yellowness.

³Statistical significance: P < 0.05.

The effects of the experimental diets on the fatty acid profile of breast muscle are presented in Table 47. A linear effect was observed for almost all the considered fatty acids. However, no quadratic effect related to HI meal utilization was observed for any of the fatty acids. A linear increase was observed for C12, C14, C15, C14:1, C16:1n7; C20:1 and Σ MUFA, with a maximum detected for the inclusion of 15% of HI meal. C15:0 showed a linear response to increasing HI meal levels ($P < 0.05$), with a maximum observed for the inclusion of 10% of HI meal. A linear decrease was instead shown for C18:0, C24:0, C18:2 n6, Σ PUFA, Σ n3 and Σ n6, with a minimum observed for the inclusion of 15% of HI meal. C20:4 n6, C22:5 n3 and C22:6 n3 showed a linear effect, with a maximum observed for the HI10 group. No significant effects were observed for the other fatty acids. Overall, the lipid health indexes, such as the Σ PUFA/ Σ SFA ratio, the Σ n3/ Σ n6 FA ratio, AI and TI, were affected negatively by dietary HI larvae meal inclusion. A linear increase was observed for all the indexes, with a maximum detected for the inclusion of 15% of HI meal.

Table 47. Effect of the dietary HI larvae meal inclusion on the fatty acid profile of breast muscle of the male broiler chickens.

Variable ²	Dietary treatments ¹				SEM	P ³	
	C	HI5	HI10	HI15		Linear	Quadratic
ΣSFA (weight % of total FA)	29.13	28.97	29.25	29.88	0.293	0.352	0.507
C12:0	0.00	0.33	0.61	1.03	0.060	<0.001	0.367
C14:0	0.23	0.43	0.55	0.74	0.032	<0.001	0.965
C15:0	0.00	0.02	0.05	0.04	0.005	<0.001	0.041
C16:0	17.63	18.24	18.74	19.24	0.20	0.002	0.887
C17:0	0.15	0.13	0.13	0.14	0.009	0.705	0.315
C18:0	9.55	8.48	7.98	7.61	0.211	<0.001	0.352
C20:0	0.19	0.24	0.23	0.24	0.011	0.152	0.245
C24:0	1.38	1.09	0.96	0.85	0.059	0.001	0.416
ΣMUFA (weight % of total FA)	27.61	30.00	31.58	32.18	0.475	<0.001	0.279
C14:1	0.00	0.03	0.09	0.14	0.045	<0.001	0.630
C16:1n-7	0.18	2.25	2.62	3.13	0.116	<0.001	0.870
C17:1	0.05	0.11	0.07	0.09	0.011	0.287	0.355
C18:1n9	25.26	27.09	28.28	28.22	0.364	0.001	0.152
C20:1	0.20	0.26	0.29	0.31	0.010	<0.001	0.330
C24:1	0.32	0.28	0.22	0.26	0.016	0.091	0.182
ΣPUFA (weight % of total FA)	42.36	40.06	37.89	36.65	0.514	<0.001	0.524
C18:2 n6	31.44	30.62	29.61	28.32	0.413	0.004	0.763
C18:3 n6	0.20	0.24	0.21	0.25	0.013	0.355	0.974
C18:3 n3	2.31	2.40	2.47	2.20	0.061	0.641	0.150
C20:2 n6	0.56	0.57	0.57	0.47	0.029	0.319	0.345
C20:4 n6	5.86	4.76	3.90	4.05	0.238	0.002	0.152
C20:5 n3 (EPA)	0.13	0.17	0.17	0.17	0.011	0.207	0.375
C22:5 n3 (DPA)	1.09	0.81	0.62	0.73	0.050	0.002	0.039
C22:6 n3 (DHA)	0.77	0.49	0.34	0.46	0.039	0.001	0.004

Others FA (weight % of total FA)	0.90	0.97	1.29	1.30	0.080	0.032	0.814
ΣPUFA/SFA	1.46	1.40	1.31	1.23	0.026	<0.001	0.901
Σn3	4.30	3.87	3.60	3.56	0.080	<0.001	0.162
Σn6	38.06	36.19	34.30	33.09	0.450	<0.001	0.646
Σn6/n3	8.93	9.39	9.63	9.40	0.127	0.140	0.169
AI	0.27	0.29	0.31	0.34	0.005	<0.001	0.957
TI	0.60	0.61	0.62	0.64	0.009	0.139	0.875

¹Four dietary treatments: C = control; HI5 = 5% inclusion level of *Hermetia illucens*; HI10 = 10% inclusion level of *Hermetia illucens*; HI15 = 15% inclusion level of *Hermetia illucens*.

²SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; FA, fatty acids; AI, atherogenicity index; TI, thrombogenicity index.

³Statistical significance: P < 0.05.

8.4.9 Microbiological findings

Salmonella spp. was not detected in neck skin samples (25g) of both C and HI animals, as specified in regulation 2073/2005/EC (EC, 2005). Similarly, *Salmonella* spp. and *Campylobacter* spp. were not identified in breast muscle samples (25g) of both C and HI birds, as specified in regulation 2073/2005/EC (EC, 2005).

8.4.10 Heavy metals and arsenic content

The concentrations of heavy metals and arsenic in the HI larva meal and breast meat samples of the broiler chickens considered in the present study are summarized in Table 48 and 49.

The arsenic, cadmium, lead and mercury levels in the HI larva meal were below the EU limits reported for animal feeds (2, 2, 10 and 0.1 mg/kg, respectively) as specified in Directive 2002/32/EC (EC, 2002), being < 0.050 (arsenic), 0.32 (cadmium), 0.069 (lead) and < 0.020 (mercury) mg/kg (Table 48). The heavy metal and arsenic contents in the breast meat sample pools of the four dietary treatments revealed no differences between the control diet (C) and the HI diets (Table 49). The level of cadmium and lead in the breast meat was below the EU limits reported for chicken meat (0.050 and 0.10 mg/kg, respectively) as specified in regulation 1881/2006/EC (modified by regulations 488/14/EU and 1005/15/EU, respectively) (EC, 2006), being < 0.050. Furthermore, the iron, nickel, copper and selenium contents in breast meat of the birds fed with the control diet (C) were higher than that of the animals fed HI meal.

Table 48. Heavy metals content in HI larvae meal.

Metal	HI meal	MRL (Dir. 2002/32/EC)
As (mg/Kg 12% h)	< 0.050	2
Cd (mg/Kg 12% h)	0.32	2
Pb (mg/Kg 12% h)	0.069	10
Hg (mg/Kg 12% h)	< 0.020	0.1
Cr (mg/Kg)	0.23	Not legislated
Fe (mg/Kg)	189	Not legislated
Ni (mg/Kg)	0.18	Not legislated
Cu (mg/Kg)	10	Not legislated
Zn (mg/Kg)	157	Not legislated
Co (mg/Kg)	< 0.050	Not legislated
Se (mg/Kg)	< 0.025	Not legislated

HI, *Hermetia illucens*.

Table 49. Heavy metals content in breast meat of the male broiler chickens.

Metal	Dietary treatments				MRL Reg. 1881/2006/EC
	C	HI5	HI10	HI15	
As (mg/Kg 12% h)	< 0.050	< 0.050	< 0.050	< 0.050	Not legislated for meat
Cd (mg/Kg 12% h)	< 0.050	< 0.050	< 0.050	< 0.050	0.050 (UE n. 488/14)
PB (mg/Kg 12% h)	< 0.050	< 0.050	< 0.050	< 0.050	0.10 (UE n. 1005/15)
Hg (mg/Kg 12% h)	< 0.020	< 0.020	< 0.020	< 0.020	Not legislated for meat
Cr (mg/Kg)	< 0.050	< 0.050	< 0.050	< 0.050	Not legislated
Fe (mg/Kg)	9.1	6.5	6.5	6.7	Not legislated
Ni (mg/Kg)	0.085	< 0.050	< 0.050	< 0.050	Not legislated
Cu (mg/Kg)	0.64	0.46	0.41	0.46	Not legislated
Zn (mg/Kg)	13	13	13	13	Not legislated
Co (mg/Kg)	< 0.050	< 0.050	< 0.050	< 0.050	Not legislated
Se (mg/Kg)	0.28	< 0.25	< 0.25	< 0.25	Not legislated

C = control; HI5 = 5% inclusion level of *Hermetia illucens*; HI10 = 10% inclusion level of *Hermetia illucens*; HI15 = 15% inclusion level of *Hermetia illucens*.

9 DISCUSSION

As a consequence of the similarities among the experimental trials, the discussion of the results is often superimposable. However, in order to make the discussion as clear as possible, some sentences will be repeated throughout the chapters.

9.1 EXPERIMENT 1

The present experimental trial is the first one, along with the experimental trial 2, which evaluated the effects of dietary full-fat TM larvae meal inclusion on growth performance, haematochemical parameters, carcass traits, histological findings and gut health of broiler chickens.

9.1.1 Growth performance

The almost 50% of the Italian male broiler chickens is generally slaughtered in the period from 50 to 60 days, in order to obtain heavy birds (3.4-3.6 kg of LW) for the production of cut-up and further processed products (Bianchi et al., 2007). The slaughter age of the present trial is also in agreement with previous studies (Sirri et al., 2016; Soglia et al., 2016).

Growth performances of the broiler chickens of the current experimental trial were consistent with the reference values recorded in the local poultry corporation in which the trial was conducted, but the final LW and overall FCR on average resulted -12.6% and +16.7% lower and higher than the Ross 708 broiler performance objectives (Aviagen, 2014). This could be related to the physical form of the feed, which was distributed in mash instead of the most frequently used crumble or pellet (Jahan et al., 2006). The body weight and feed intake of the birds in the present experiment improved with increasing levels of TM meal inclusion, but the feed efficiency resulted impaired. Data regarding the TM meal utilization in poultry are limited and sometimes controversial. Ramos-Eldoury et al. (2002) showed no effects for the growth performance in fast-growing chickens fed sorghum-soybean meal-based diets in which the full-fat TM inclusion level ranged from 5 to 10% in partial substitution of soybean meal and vegetable oil. On the contrary, Bovera et al. (2015 and 2016) found improved growth performance in fast-growing chickens fed on a corn-soybean meal-based diets in which the 29.6% of soybean meal was completely replaced by TM meal. In the current experimental trial, three different increasing levels of TM meal inclusion were tested in a corn-soybean-gluten meal-based diet replacing a portion of the dietary high-protein feedstuff sources (i.e., soybean and gluten meal) and soybean oil. In the present experiment, the body weight of the birds fed TM diets increased with increasing feed intake. Even if a feed-choice test was not performed, the increase of the average DFI

in the birds may suggest an improvement of the diet palatability related to TM meal inclusion level. To the best of the authors' knowledge, no feed-choice trial has been performed to test the dietary inclusion of insect meal in broiler chickens. However, Cullere et al. (2016) performed a feed-choice test in broiler quails and observed that the birds tended to prefer the diets including *Hermetia illucens* meal. This may be related to the innate behavior of chickens, which naturally consume insects when reared in free-range systems (Zuidhof et al., 2003). However, the increase of the body weight and feed intake resulted in an overall impairment of the feed efficiency in the birds fed TM diets. Different hypotheses can be formulated to explain this negative effect. Firstly, the detrimental effect on FCR may be caused by the chitin content of TM diets, which has been reported to negatively influence the nutrient digestibility of crude protein (Razdan and Pettersson, 1994; Hossain and Blair, 2007) and organic matter (Razdan and Pettersson, 1994). Ravindran and Blair (1993) also pointed out that the chitin of insects is difficult to digest by domestic poultry. All these authors found no effects on growth performance of chickens fed diets supplemented with commercial sources of chitin instead of the whole insect of the current study (Razdan and Petterson, 1994; Hossain and Blair, 2007), but the chitin form may have influenced the results. Furthermore, the crude protein digestibility of TM meal used in the current trial was moderate (0.60), as reported by De Marco et al. (2015). Moreover, to the best of the authors' knowledge, this is the first study in which broiler chickens were fed diets with TM larvae meal from the hatch to the slaughter age. Indeed, the TM meal administration of previous studies started from 7 (Ramos-Eldoruy et al., 2002; Ballitoc and Sun, 2013) and 30 (Bovera et al., 2015) days of age, respectively, with an inclusion rate ranging from 0.5 (Ballitoc and Sun, 2013) to 29.6% (Bovera et al., 2015).

9.1.2 Haematological and serum parameters

The haematological and serum biochemical parameters give information about health status of the birds (Lumej, 2008; Salamano et al., 2010; De Marco et al., 2013). The results of the present experimental trial suggest that dietary TM meal inclusion did not impair these blood parameters, thus confirming the safety of TM utilization in poultry diets, as previously observed by Bovera et al. (2015). Furthermore, all the blood parameters obtained in the present study fell within the physiological ranges (Lumej, 2008).

9.1.3 Carcass characteristics

Dietary TM meal inclusion did not influence the carcass characteristics of the birds of the present experimental trial. This is in agreement with Bovera et al. (2016), which did not find any differences for the same considered parameters. A similar figure was given by Hwangbo et al. (2009) and Cullere et

al. (2016), who did not observe any effects after house-fly maggots and black soldier fly meal inclusion, respectively, in the diet of broiler chickens and quails. On the contrary, Ballitoc and Sun (2013) found improved slaughter, dressed carcass and eviscerated weights in broiler chickens fed TM diets.

9.1.4 Histopathological findings

Dietary TM meal inclusion did not affect the development or severity of the histopathological features of the birds of the present experimental trial, thus suggesting no negative influence on animal health. However, the broiler chickens of the current experiment showed different degrees of lymphoid system activation. This result could be related to the stress occurrence in modern poultry rearing operations. Stress can be caused by a variety factors, physiological (rapid growth rate) and social (overcrowding) ones included (Liles et al., 2015). However, a great deal of individual bird variability in some immunological measures (i.e., stimulation index, heterophils to lymphocyte ratio and lymphocyte blastogenesis) may also be considered (Talebi et al., 1995).

9.1.5 Gut mucosal morphology

Dietary TM meal inclusion partially affected the intestinal morphology of the broiler chickens of the current experimental trial. In particular, shorter villi, deeper crypts and reduced Vh/Cd were observed in the birds fed with 15% level of TM inclusion compared to the animals fed with TM5 and control diets, respectively. The small intestinal morphology, especially regarding crypts and villi of the absorptive epithelium, plays a key role in the final phase of nutrient digestion and assimilation (Wang and Pen, 2008). The physiological gut morphological development appears to be characterized by the identification of long villi and shallow crypts. Indeed, longer villi are generally associated with increased total luminal absorptive area and subsequent satisfactory digestive enzyme action and higher transport of nutrients (Laudadio et al., 2012). Simultaneously, shallower crypts reflect the prolonged survival of villi without the need for renewal (Oliveira et al., 2008), with reduced energy expenditure for this process and consequent enabled growth of other tissues (Miles et al., 2006). On the contrary, lower villus heights and greater crypt depths may lead to poor digestion and less absorption of nutrients and, as a consequence, poor performance of the animals (Qaisrani et al., 2014). Modifications of protein source and dietary structure has already been reported to negatively influence small intestinal morphology in broilers in terms of decreased Vh and increased Cd (Qaisrani et al., 2014). Therefore, the altered morphometric indices observed in male broilers fed with 15% level of TM meal inclusion could be related to the higher level of insects of this diet, thus also suggesting that lower levels may be preferable. A similar consideration can also be applied for explaining the

growth performance observed in the current experiment. Indeed, the overall FCR increased linearly with increased levels of TM meal, reaching its maximum with the inclusion of 15% of mealworms. The LW in the finisher period also increased quadratically till 5% level of TM meal inclusion, decreasing up to the inclusion of 15% of insects. Furthermore, the alterations of intestinal morphology can also justify the observed growth performance. Indeed, since it is well known that rapid growth of chickens directly depends on morphological and functional integrity of the digestive tract (Wang and Peng, 2008), the relationship between the altered gut morphometric indices and the worsening of the growth performance seems reasonable.

Apart from TM meal utilization, the current experimental trial confirms that morphometric indices followed a proximodistal decreasing gradient from the duodenum to the ileum, as previously reported (Iji et al., 2001; Forder et al., 2007; Murakami et al., 2007). This is related to the intestinal absorption processes, which evolved differently depending on the considered segment. Indeed, the duodenum is the intestinal tract with the fastest cell renewal, and is also the first segment of the small intestine to receive physical, chemical and hormonal stimuli provoked by the presence of the diet in the lumen (Macari, 1998). Furthermore, the jejunum is an important site for nutrient digestion (Iji et al., 2001).

9.1.6 Gut mucin composition

The broiler chickens fed with 5% level of TM meal inclusion of the current experimental trial showed greater mucin staining intensity than those fed with 10 and 15% levels in the intestinal crypts. Forder et al. (2007) pointed out that bacteria may influence the mucin production. In particular, some bacteria are known to possess mucin-specific glycosulfatases, which can cleave sulfate from its linkage to mucin sugars (Robertson and Wright, 1997). As colonization becomes greater, the chicken gut needs for greater protection against mucus degradation and the increase in sialomucins production can be observed (Forder et al., 2007). However, no mucin-degrading bacteria were found in the cecal microbiota of TM5 birds (see chapter 9.1.7). Therefore, the increase in mucin staining intensity observed in these animals could be related to TM meal utilization, rather than changes in bacterial colonization. Since mucins constitute a digestion- and absorption-assisting medium and act as a substrate for the resident flora and by aggregation facilitate the removal of pathogenic bacteria (Forstner and Forstner, 1994), this finding may have important implication. Indeed, the utilization of TM meal at low inclusion rates (i.e., 5%) could be preferable to preserve the protective properties of mucin glycoproteins in order to optimize the digestive process and to prevent the enteric infections. Independently of TM meal utilization, the intestinal crypts of the birds of the current trial showed greater acidic sialylated mucins staining intensity. Similarly, higher acidic sialylated mucin staining intensity than acidic sulfated was observed in the intestinal villi. This represents an interesting finding, since sialic acid groups have protective properties (Corfield et al., 1992) and increase in acidic

sialylated mucins production has been hypothesized to represent a defense strategy against mucus degradation by bacterial colonization (Forder et al., 2007).

The intestinal crypts of both C and TM animals of the present research showed lower mucin staining intensity in the caecum compared with the other gut segments. This finding is in agreement with what observed by Tsirtsikos et al. (2012a), even if the authors provided no explanation for that result. The avian caecum has been found to be a site for fermentation and further digestion of feed, for utilization and absorption of water and nitrogenous components, for microbial action of both beneficial and disease-causing organism, and as a site for production of immunoglobulins and antibodies (Clench and Mathias, 1995). As previously reported mucins enhance the propulsion of chyme, modulate nutrient absorption and protect the intestinal mucosa from enteric pathogens (Forstner and Forstner, 1994). Since the small intestine represents the major site of nutrient absorption (Dingle, 1991) and the ileum has been suggested to be more predisposed to bacterial colonization (Forder et al., 2007), it is possible to speculate that the mucin synthesis for nutrient metabolism and gut protection could be reduced in the caecum. Furthermore, because the caecum is blind-ended, its contents can be retained for long periods, with no need for mucin secretion to facilitate the propulsion of chyme. Therefore, the decreased mucin staining intensity in the caecal crypts observed in the present experiment could be considered a physiological feature related to the different anatomy and physiology of the caecum.

Independently of dietary TM meal inclusion, the intestinal villi of the broiler chickens of the present experimental trial showed greater mucin staining intensity in the ileum compared with the other gut segments. This is consistent with previous findings in chickens (Uni et al., 2003; Forder et al., 2007), which demonstrated a distal increase in the density of goblet cells along the duodenal-ileal axis (Uni et al., 2003). Forder et al. (2007) suggested that the distal ileum could be a preferred region for bacterial colonization, thus explaining the need for greater protection and subsequent higher mucin synthesis.

The intestinal crypts of both C and TM birds of the current research showed greater mucin staining intensity in the base compared with the other crypt fragments. This is in agreement with previous studies, in which the decreased stain in the crypt tip has been suggested to be related to the process of proliferation and maturation of goblet cells (Uni et al., 1998; Tsirtsikos et al., 2012a). Similarly, the intestinal villi showed higher mucin staining intensity in the base compared with the other villus fragments. This is in contrast with what observed by Tsirtsikos et al. (2012a and 2012b), which found higher staining intensity at the villus tip and explained this increased accumulation as a confirm for the key role of mucin in the protection of gut epithelium against luminal threats (Tsirtsikos et al., 2012a and 2012b). However, the process of cell proliferation in chicken intestinal epithelium has also been reported to occur along the entire length of the villus, with proliferation activity decreasing from crypt to the upper half of the villus (Uni et al., 1998). Therefore, the higher mucin staining intensity observed at the villus base in the birds of the present experimental trial can be related to the physiological goblet cells proliferation process occurring in villus compartment.

9.1.7 Cecal microbiota characterization

In the broiler chickens of the present experimental trial, cecal microbiota was mainly colonized by the phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, as previously reported in normal chickens (Wise and Siragusa, 2007; Tillman et al., 2011; Wei et al., 2013; Oakley et al., 2014). However, the predominance of the phylum *Bacteroidetes* over *Firmicutes* is in contrast with these studies. The results of the current trial are more in agreement with what observed in duck (Vasaï et al., 2014) goose (Xu et al., 2017) and turkey (Scupham et al., 2008), where the dominant phyla, from high to low, have been reported to be *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. It is well known that chickens and ducks employ roughage utilization (Wang et al., 2017) and *Bacteroidetes* members are involved in the digestion of complex polysaccharides (Lan et al., 2006). However, the influence of multiple factors on gut microbial diversity (including heredity and environment) always needs to be taken into account (Wang et al., 2017). The identification of decreased abundance of *Firmicutes* and lower *Firmicutes*:*Bacteroidetes* ratios in the birds fed with TM15 when compared to C diet of the current experimental trial represents a potential negative finding. Indeed, bacteria within *Firmicutes* phylum have an important role in the digestion of feed and the host health (Li et al., 2016a). Furthermore, greater *Firmicutes*:*Bacteroidetes* ratios have been associated with bacterial profile with higher capacity of energy harvesting (Singh et al., 2012).

The current experiment observed *Bacteroides*, *Clostridium*, *Alistipes* and *Coprobacter* as the most predominant genera in the cecal microbiota of the broilers chickens. The majority of studies reported that the most predominant genera found in chicken cecum are *Clostridium*, *Ruminococcus*, *Lactobacillus* and *Bacteroides* (Gong et al., 2007; Stanley et al., 2009; Danzeisen et al., 2011; Wei et al., 2013; Costa et al., 2017), with *Alistipes* genus also being represented by > 1% of the bacterial sequences (Wei et al., 2013). However, the identification of *Coprobacter* genus represents an unexpected finding. Indeed, this genus is highly prevalent in human intestinal microbiota and is characterized by the ability to produce propionic acid, lower guanine and cytosine content and composition of its major fatty acids (Shkoporov et al., 2013). The current research is the first one to report *Coprobacter* genus in chicken microbiota.

In the present experimental trial, the broiler chickens fed with TM showed a decrease in the abundance of *Clostridium*, *Coprococcus*, *L-Ruminococcus* and *Ruminococcus* genera in their cecal community. As already reported, *Clostridium* and *Ruminococcus* are some of the most predominant genera found in chicken cecum (Gong et al., 2007; Wei et al., 2013; Danzeisen et al., 2011). *Clostridium* and *Coprococcus* genera also encompass bacteria capable of producing butyric acid (Pryde et al., 2002; Yang et al., 2017), which has a positive role on growth performance, intestinal villus structure and pathogen control, along with anti-inflammatory properties (Onrust et al., 2015). Furthermore, *Ruminococcus* can also produce other short chain fatty acids (i.e., acetic and succinic acid) through glucose metabolism and digest cellulose in food (Liu et al., 2008). Short chain fatty

acids production is of vital importance for intestinal health, since they are a remarkable source of energy for enterocytes (Sunkara et al., 2012) and can suppress gut pathogens (Rehman et al., 2007). The decrease of the above mentioned bacterial taxa suggests that TM meal utilization may negatively modulate the cecal microbiota of birds. Another interesting aspect to consider in relation to this negative modulation is that the depletion of *Ruminococcus* genus was particularly evident in the broilers fed with TM10 and TM15 diets. Furthermore, TM15 birds showed decreased abundances of *Firmicutes* phylum and reduced *Firmicutes:Bacteroidetes* ratios. As already described before (see chapter 9.1.5), the birds fed with 15% level of TM meal inclusion showed the worst gut morphology in terms of shorter villi, deeper crypts and reduced villus height to crypt depth ratios, suggesting that lower insect levels (i.e., 5%) could be preferable. Therefore, the gut microbiota of the same animals seems to partially confirm this hypothesis. Interestingly, the broilers fed with TM15 diet also displayed the worst growth performance in terms of highest feed conversion ratios (see chapter 9.1.1). Even if a direct cause-effect relationship between diversity and composition of cecal microbiota and bird performance has not yet been confirmed, the results of gut morphology, mucin composition and microbiota taken together suggest a potential influence of the intestinal features on the growth of animals.

9.2 EXPERIMENT 2

The present experimental trial is the first one, along with the experimental trial 1, which evaluated the effects of dietary full-fat TM larvae meal inclusion on growth performance, haematochemical parameters, carcass traits, histological findings and gut health of broiler chickens.

9.2.1 Growth performance

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

The body weight, weight gain and feed intake of the birds in the present experiment improved with increasing levels of TM meal inclusion, but the feed efficiency resulted partially impaired. Little information on the influence of dietary TM meal inclusion in broiler chickens is currently available. Ramos-Eldoury et al. (2002) did not show any effects for the growth performance in fast-growing chickens fed diets in which the TM inclusion level ranged from 50 to 100 g/kg. Differently, Ballitoc and Sun (2013) and Bovera et al. (2015) observed improved growth performance in fast-growing chickens fed diets with low (from 5 to 100 g/kg) or high (296 g/kg) TM inclusion levels, respectively. Similar findings were obtained by Loponte et al. (2017) in partridges fed diets in which the TM inclusion level ranged from 250 to 500 g/kg. Recently, Islam and Yang (2017) also found a positive effect of a

mealworm-based probiotic on broiler growth performance. Some authors also explored the possibility to use other insect meals in poultry feeding. Adeniji (2007) and Hwangbo et al. (2009) studied the effects of housefly-maggots as feed supplement in the diet of broiler chickens: the first found no differences for growth performance with inclusion levels ranging from 55 to 220 g/kg, while the latter (inclusion rate: 50-200 g/kg) observed a linear increase in LW gain. Ijaiya and Eko (2009) evaluated the effects of replacing dietary fishmeal with silkworm meal (inclusion rate: 22-93 g/kg) on growth performance of broiler chickens, finding no differences related to insect meal utilization. Also Oyegoke et al. (2006) and Wang et al. (2005) observed no adverse effects on growth performance of broiler chickens fed diets with *Cirina forda* (inclusion rate: 20-40 g/kg) and *Gryllus testaceus* (inclusion rate: 50-150 k/kg), respectively. Cullere et al. (2016) recently studied the influence of the inclusion (from 100 to 150 g/kg) of *Hermetia illucens* meal in quail diet, finding no differences for growth performances. Schiavone et al. (2017b) also evaluated the effects of replacing soybean oil with *Hermetia illucens* fat (substitution rate: 50-100%) on growth performance of broiler chickens, finding no differences related to insect fat utilization. The wide variability of the results obtained in the previous studies may be related to the nutritive value of the insect meal used, which can be influenced by the species, the insect life stage (adult, larva or pupa) and the insect rearing substrate (Sánchez-Muros et al., 2014).

The improvement of feed intake observed in the birds fed TM diets of the present research was considered suggestive of increased feed palatability in relation to the addition of yellow mealworms, since insects are naturally consumed by wild birds and free-range poultry (Zuidhof et al., 2003). In particular, the increased DFI observed in the starter period, which was accompanied by increased LW and ADG and unaffected FCR, was quite relevant. Indeed, starter period (from hatch to 10 days) is considered the most important in broiler production, since growth and development take place at an incredible rate during it (Aviagen, 2014). In this period, the chicks' weight quadruples, thus influencing the following growth rate (Aviagen, 2014). On the contrary, the increased DFI observed in the growing period was accompanied by unaffected LW and ADG and subsequently impaired FCR, thus representing a negative effect related to TM meal utilization. De Marco et al. (2015) speculated that the chitin contained in the exoskeleton of the TM meal may negatively influence the apparent digestibility coefficient of the total tract of nutrients. Furthermore, Ravindran and Blair (1993) pointed out that the chitin of insects is difficult to digest by domestic poultry. As suggested by Rumpbold and Schlüter (2013), the partial chitin removal through high pressure processing could improve the use of insects as feeding ingredient thanks to disruption of the link between some chitin-bound proteins.

9.2.2 Haematological and serum parameters

All the blood parameters evaluated in the present experimental trial fell within the physiological ranges (Lumej, 2008), thus suggesting that TM meal utilization does not affect health status of the animals.

As already observed by Bovera et al. (2015), the majority of the haematochemical and serum biochemical traits were not affected by yellow mealworm inclusion in the birds of the present experiment. Similar findings were obtained by Schiavone et al. (2017b) in broilers fed diets with *Hermetia illucens* fat. However, the erythrocytes increased and albumin and GGT decreased with increasing levels of TM meal utilization. Loponte et al. (2017) also observed lower albumin when TM and *Hermetia illucens* meals were included in the diets of partridges. Interestingly, this finding was accompanied by the increase of albumin/globulin ratio, which was also reported by Bovera et al. (2015) and ascribed to the properties of chitin contained in insect meal. High globulin concentrations and low albumin/globulin ratios generally indicate better disease resistance and immune response of birds (Griminger and Scanes, 1986). Another interesting finding is represented by the decrease of GGT serum levels in TM animals. Indeed, a high GGT concentration in birds is used as an indicator of liver disease and bile flow disorders (Ognik and Krauze, 2016). Therefore, GGT reduction can be considered a positive effect related to TM meal utilization.

9.2.3 Carcass characteristics

The majority of the carcass traits of the broilers in the present experimental trial were not influenced by dietary TM meal inclusion, as previously observed by Bovera et al. (2016). Similar findings were obtained by Cullere et al. (2016) and Schiavone et al. (2017b) 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 inclusion. 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 these authors, 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 mealworm utilization may improve fat mass in broiler chickens (USDA, 2011).

9.2.4 Histopathological findings

As already observed for the male broiler chickens of the experimental trial 1, dietary TM meal inclusion did not affect the development or severity of the histopathological features of the birds of the present experiment, thus suggesting no negative influence on animal health. Similarly to the animals

of the experimental trial 1, the broiler chickens of the current experiment showed different degrees of lymphoid system activation. As previously explained (see chapter 9.1.4), this result could be related to the stress occurrence in modern poultry rearing operations (Liles et al., 2015).

9.2.5 Gut mucosal morphology

Dietary TM meal inclusion did not influence the gut morphology of the birds of the present experimental trial. It is well known that morphometric measurements of Vh and Cd are generally used to assess intestinal development (Franco et al., 2006), since represent useful indicators of gut proliferative and absorptive compartments (Lenhardt and Mozes, 2003). The Vh/Cd ratio is also evaluated, because it gives an indication of the likely maturity and functional capacity of the enterocytes (Hampson, 1986). As already observed for the male broiler chickens of the experimental trial 1, the present research confirms that both duodenum and jejunum show a greater morphological development compared with the ileum (Uni et al., 1999; Iji et al., 2001). Therefore, dietary TM inclusion preserves a proximodistal decreasing gradient of the morphometric indexes from the duodenum to the ileum, thus suggesting the maintenance of the physiological gut development (Iji et al., 2001; Forder et al., 2007; Murakami et al., 2007).

9.2.6 Gut mucin composition

The broiler chickens fed with 5% level of TM meal inclusion of the current experimental trial showed greater mucin staining intensity than those fed with 10 and 15% levels in the intestinal villi. Furthermore, lower mucin staining intensity was found in the intestinal villi of the birds fed with 10% level of TM meal inclusion in comparison with C diet. Forder et al. (2007) pointed out that bacteria may influence the mucin production. In particular, bacteria such as *Helicobacter pylori* possess the enzymatic ability to disrupt the oligomeric structure of mucin, enabling the pathogen to move freely in the mucus layer, assisted by its highly active flagellum and its ability to down-regulate mucin synthesis (Deplancke and Gaskins, 2001). As bacterial colonization becomes greater, the chicken gut needs for greater protection against mucus degradation and the increase in sialomucins production can be observed (Forder et al., 2007). Interestingly, the birds of the present study showed a high abundance of *Helicobacter* genus in their cecal microbiota, with higher percentages observed in the broilers fed with TM diets and the maximum ones quite identified in the birds fed with 10% inclusion level (see chapter 9.2.7). Therefore, it is possible to hypothesize that the utilization of TM meal at low inclusion rates (i.e., 5%) may be useful to enhance mucin synthesis as protection strategy when bacteria-induced mucolysis occurs.

Independently of TM meal utilization, the intestinal crypts of the birds of the current trial showed greater neutral mucin staining intensity. The production of neutral mucins has been reported to serve as a protective mechanism against invasion by pathogenic bacteria (Dean-Nystrom and Samuel, 1994) and to increase intestinal maturity in order to facilitate the breakdown of complex carbohydrates (Forder et al., 2007). On the contrary, similar staining intensity of the three types of mucins was observed in the intestinal villi. The variations in the proportion of the goblet cells containing the mucin types observed in the intestinal crypts instead of villi could be related to the higher presence of mucin-producing goblet cells in the first compartment, as reported by Uni et al. (1998).

As already reported for the male broiler chickens of the experimental trial 1, the intestinal crypts of both C and TM animals of the present research showed lower mucin staining intensity in the caecum compared with the other gut segments. This finding is in agreement with what observed by Tsirtsikos et al. (2012a) and could be related to the peculiar anatomy and physiology of the caecum (see chapter 9.1.6).

Independently of dietary TM meal inclusion, the intestinal villi of the broiler chickens of the present experiment showed greater mucin staining intensity in the ileum compared with the other gut segments. This is similar to what observed for the male broiler chickens of the experimental trial 1 and is consistent with previous findings in chickens (Uni et al., 2003; Forder et al., 2007), which described different mucin dynamics depending on the gut segment considered (see chapter 9.1.6).

As already reported for the male broiler chickens of the experimental trial 1, both intestinal crypts and villi of either C or TM birds of the current research showed greater mucin staining intensity in the base compared with the other fragments. Crypt findings are in agreement with previous studies (Uni et al., 1998; Tsirtsikos et al., 2012a), while contrasting results have previously been reported for villi (Tsirtsikos et al., 2012a and 2012b). However, both findings can be explained in relation to the physiological goblet cells proliferation and maturation processes (see chapter 9.1.6).

9.2.7 Cecal microbiota characterization

In the broiler chickens of the present experimental trial, cecal microbiota was mainly colonized by the phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, as previously reported in normal chickens (Wise and Siragusa, 2007; Tillman et al., 2011; Wei et al., 2013; Oakley et al., 2014). However, the predominance of the phylum *Bacteroidetes* over *Firmicutes* is in contrast with these studies. The results of the current research, which are similar to those reported for the male broiler chickens of the experimental trial 1, are more in agreement with what observed in duck (Vasaï et al., 2014) goose (Li et al., 2017) and turkey (Scupham et al., 2008), where the dominant phyla, from high to low, have been reported to be *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. It is well known that chickens and ducks employ roughage utilization (Wang et al., 2017) and *Bacteroidetes* members have a key role in the digestion of complex polysaccharides (Lan et al., 2006). However, the influence of multiple factors

on gut microbial diversity (including heredity and environment) always needs to be considered (Wang et al., 2017). The identification of increased abundance of *Firmicutes* and higher *Firmicutes:Bacteroidetes* ratios in the birds fed with TM5 when compared to TM10 and TM15 diets, respectively, of the current experimental trial suggests a potential better gut microbiota modulation by lower insect levels. As already mentioned before, bacteria within *Firmicutes* phylum have an important role in the digestion of feed and the host health (Li et al., 2016a). Greater *Firmicutes:Bacteroidetes* ratios have also been associated with bacterial profile with higher capacity of energy harvesting (Singh et al., 2012).

The current experiment observed *Bacteroides*, *Helicobacter*, *Clostridium*, *Alistipes*, *Coprobacter* and *Ruminococcus* as the most predominant genera in the cecal microbiota of the broilers chickens. The majority of studies reported that the most predominant genera found in chicken cecum are *Clostridium*, *Ruminococcus*, *Lactobacillus* and *Bacteroides* (Gong et al., 2007; Stanley et al., 2009; Danzeisen et al., 2011; Wei et al., 2013; Costa et al., 2017), with *Alistipes* genus also being represented by > 1% of the bacterial sequences (Wei et al., 2013). However, the percentages related to *Coprobacter* and *Helicobacter* genera particularly stand out. Firstly, the identification of *Coprobacter* genus represents an unexpected finding. Indeed, this genus is highly prevalent in human intestinal microbiota and is characterized by the ability to produce propionic acid, lower guanine + cytosine content and composition of its major fatty acids (Shkoporov et al., 2013). The current research, along with the experimental trial 1, is the first one to report *Coprobacter* genus in chicken microbiota. Secondly, high abundance of *Helicobacter* genus in chicken cecum has been reported to be related to its potential to remove hydrogen, which could benefit the other members of the microbial community and the host's ability to recover energy from food (Sergeant et al., 2014). Another important aspect to consider is that some specific enterohepatic *Helicobacter* species (i.e., *Helicobacter pullorum*) can be found in the gut of healthy chickens as well as in liver and intestine of hens with vibronic-like liver lesions and humans with gastroenteritis (Stanley et al., 1994). However, no *Helicobacter pullorum* was identified in the birds of the current trial. Another important aspect that needs to be underlined is that the detection of high abundance of *Helicobacter* genus cannot be excluded as potential cause of mucin synthesis reduction in TM10 birds (see chapter 9.2.6), because of *Helicobacter pylori* (which was identified in the broiler chickens of the current research) mucolytic activity (Deplancke and Gaskins, 2001).

In the present experimental trial, the broiler chickens fed with TM showed an increase in the abundance of *Clostridium*, *Sutterella* and *Alistipes* genera in their cecal community. *Clostridium* genus is one of the most predominant members found in chicken cecum (Gong et al., 2007; Wei et al., 2013; Danzeisen et al., 2011; Costa et al., 2017) and it also encompasses bacteria capable of producing butyric acid (Pryde et al., 2002), which has a positive role on growth performance, intestinal villus structure and pathogen control, along with anti-inflammatory properties (Onrust et al., 2015). In parallel, *Alistipes* bacteria have been reported to be bile-resistant organism with the ability to produce fibrinolysin, digest gelatin and ferment carbohydrate to produce acetic acid, thus making them

considered as gut beneficial bacteria (Rautio et al., 2003). Another interesting finding of the cecal community of the birds fed with TM in the current research is the identification of increased abundance of *Sutterella* genus, which has been reported to be a potential probiotic present in pigeon “milk” that can improve the rate of growth and feed conversion ratio in chickens (Gillespie et al., 2012). The increase of the above mentioned bacterial taxa suggests that TM meal utilization may positively modulate the cecal microbiota of birds. On the contrary, the reduction of *Ruminococcus* genus within the cecal community of the broiler chickens fed with TM of the present experiment may be considered a potential negative finding. Indeed, *Ruminococcus*, apart from being one of the most predominant members found in chicken cecum (Gong et al., 2007; Stanley et al., 2009; Danzeisen et al., 2011; Wei et al., 2013; Costa et al., 2017), can also produce short chain fatty acids (i.e., acetic and succinic acid) through glucose metabolism and digest cellulose in food (Liu et al., 2008). It is well known that short chain fatty acids production is of vital importance for intestinal health, since they are a remarkable source of energy for enterocytes (Sunkara et al., 2012) and can suppress gut pathogens (Rehman et al., 2007).

9.3 EXPERIMENT 3

The present experimental trial is the first one which evaluated the effects of dietary full-fat TM larvae meal inclusion on growth performance, haematochemical parameters, carcass traits, histological findings, gut health and meat quality of free-range chickens.

9.3.1 Growth performance, haematological and serum parameters and carcass characteristics

Dietary TM meal inclusion did not affect the growth performance, haematological and serum parameters or carcass characteristics of the free-range chickens of the present experimental trial. These results confirm that TM can be used safely in poultry diets. Ramos-Elorduy et al. (2002) 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 and carcass traits of the broilers, because of the improved final body weight, feed intake, feed efficiency and 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, carcass characteristics or blood traits of the birds, except for uric acid, albumin/globulin ratio, AST and ALT. In

another study, it was pointed out that TM could be successfully used to replace 4% soybean meal in laying hens diets (Wang et al., 2015).

9.3.2 Histopathological findings

In the present experimental trial, dietary TM meal inclusion did not induce histopathological changes, thus suggesting no negative influence on animal health. Glandular stomach alterations were considered paraphysiological because, in the authors' opinion, they were probably related to the free-range farming system. Indeed, outdoor access entails less control of animal feeding, with the possible ingestion of foreign bodies. However, the activation of the lymphoid tissue, in both dietary treatments, represents the most interesting result. It has been reported that keeping birds in free-range systems may favor the occurrence of gastrointestinal parasitic diseases, with a high prevalence of coccidiosis and helminthiasis (Magwisha et al., 2002; Tomza-Marciniak et al., 2014). Despite all the birds being vaccinated against coccidiosis, the immunological variation between strains of the same species might not have conferred total protection (Chapman, 2014). Furthermore, the chronic inflammation 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, observing 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.

9.3.3 Gut mucosal morphology

Similarly to what observed for the female broiler chickens of the experimental trial 2, dietary TM meal inclusion did not affect the morphology of the small intestine of the free-range chickens of the current research, thus suggesting no influence on nutrient metabolization or performance with 7.5% level of inclusion. The greater development of the duodenum, as already reported for the broiler chickens of the experimental trials 1 and 2, is in agreement with the results of Uni et al. (1999), Kondo (2003) and Murakami et al. (2007).

9.3.4 Gut mucin composition

Dietary TM meal inclusion in free-range chickens of the current experimental trial did not influence the mucin staining intensity either in the intestinal crypts or villi. This finding could reflect an innate feeding habit, since chickens naturally consume insects when reared in free-range systems (Zuidhof et al., 2003).

Independently of TM meal utilization, the intestinal crypts of the birds of the current experiment showed greater acidic sialylated mucin staining intensity. This is similar to what reported for the male broiler chickens of the experimental trial 1 and represents an interesting finding in terms of gut protection (see chapter 9.1.6). On the contrary, similar staining intensity of the three types of mucins was observed in the intestinal villi, thus probably reflecting the higher presence of mucin-producing goblet cells in crypts (Uni et al., 1998).

As already reported for the broiler chickens of the experimental trials 1 and 2, the intestinal crypts of both C and TM animals of the present research showed lower mucin staining intensity in the caecum compared with the other gut segments. This finding is in agreement with what observed by Tsirtsikos et al. (2012a) and could be related to the peculiar anatomy and physiology of the caecum (see chapter 9.1.6).

Independently of dietary TM meal inclusion, the intestinal villi of the free-range chickens of the present experiment showed greater mucin staining intensity in the ileum compared with the other gut segments. This is similar to what observed for the broiler chickens of the experimental trials 1 and 2 and is consistent with previous findings in chickens (Uni et al., 2003; Forder et al., 2007), which described different mucin dynamics depending on the gut segment considered (see chapter 9.1.6).

As already reported for the broiler chickens of the experimental trials 1 and 2, both intestinal crypts and villi of either C or TM birds of the current research showed greater mucin staining intensity in the base compared with the other fragments. Crypt findings are in agreement with previous studies (Uni et al., 1998; Tsirtsikos et al., 2012a), while contrasting results have previously been reported for villi (Tsirtsikos et al., 2012a and 2012b). However, all these findings can be explained in relation to the physiological goblet cells proliferation and maturation processes (see chapter 9.1.6).

9.3.5 Cecal microbiota characterization

The cecal microbiota of the free-range chickens of the present experimental trial showed higher OTUs richness and alpha diversity when fed with TM diet compared to control feed. This represents a quite relevant finding, since high levels of diversity have been reported to help maintain the stability of intestinal microbiota after environmental stress (Xue et al., 2015) and determine the colonization resistance against invading pathogens (Kuhn et al., 1993). Another interesting aspect to consider is that the average Shannon index identified in the current experiment was about 8. This is in contrast

with the previous studies about the chicken cecal microbiota, which reported a Shannon index varying from 3 to 6 (Stanley et al., 2013; Sergeant et al., 2014). Mancabelli et al. (2016) already pointed out that free-range chickens displayed a higher level of complexity of the gut microbiota compared to that found in broilers, with a Shannon index slightly higher than 6. Interestingly, Ferrario et al. (2017) also observed similar microbial diversity between free-range and feral (i.e., formerly domesticated, wild-living) chickens, with the last being characterized by a Shannon index around 7. Therefore, it could be hypothesized that the semi-wild and wild rearing conditions may progressively modulate the complexity of the intestinal microbiota, thus underlying the role of environment and human influence on the bacterial communities within the chicken gastrointestinal tract.

In the free-range chickens of the present research, cecal microbiota was mainly colonized by the phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, as previously reported in normal chickens (Wise and Siragusa, 2007; Tillman et al., 2011; Wei et al., 2013; Oakley et al., 2014). The predominance of the phylum *Bacteroidetes* partially agrees with Mancabelli et al. (2016), which observed a comparative reduction of *Firmicutes* and concomitant increase of *Bacteroidetes* and *Proteobacteria* in free-range chickens compared to broilers. The identification of increased abundance of *Firmicutes*, decreased abundance of *Bacteroidetes* and higher *Firmicutes*:*Bacteroidetes* ratios in the birds fed with TM of the current experimental trial is a quite relevant finding. Indeed, bacteria within *Firmicutes* phylum have an important role in the digestion of feed and the host health (Li et al., 2016a).

Furthermore, greater *Firmicutes*:*Bacteroidetes* ratios have been associated with bacterial profile with higher capacity of energy harvesting (Singh et al., 2012).

Differently from phyla, at genus level there is some controversy over the predominant taxa in chicken cecal microbiota. The majority of studies reported that the most predominant genera found in chicken cecum are *Clostridium*, *Ruminococcus*, *Lactobacillus* and *Bacteroides* (Gong et al., 2007; Stanley et al., 2012; Danzeisen et al., 2011; Wei et al., 2013; Costa et al., 2017), while Callaway et al. (2009) also identified *Prevotella* as the most abundant genus. The current experiment, which observed *Bacteroides*, *Clostridium* and *Ruminococcus* as the most predominant genera, can fit into the overall view of these previous studies.

In the present research, the free-range chickens fed with TM showed an increase in the abundance of *Clostridium*, *Oscillospira*, *Ruminococcus*, *Coprococcus* and *Sutterella* genera in their cecal community. As already pointed out, *Clostridium* and *Ruminococcus* are some of the most predominant genera found in chicken cecum (Gong et al., 2007; Wei et al., 2013; Danzeisen et al., 2011). Therefore, TM meal utilization may favor the preservation of the physiological cecal community. On the other hand, *Clostridium* genus, along with *Oscillospira* and *Coprococcus*, also encompasses bacteria capable of producing butyrate (Pryde et al., 2002; Yang et al., 2017). Butyrate has been demonstrated to have a positive role on growth performance, intestinal villus structure and pathogen control, as well as anti-inflammatory properties (Onrust et al., 2015). Furthermore, bacteria belonging to *Ruminococcus* genus can also produce other short chain fatty acids (i.e., acetic and succinic acid) through glucose metabolism and digest cellulose in food (Liu et al., 2008). It is well

known that short chain fatty acids are an important source of energy for enterocytes and are vital for intestinal health (Sunkara et al., 2012). Another interesting finding of the cecal community of the birds fed with TM in the current experimental trial is the identification of increased abundance of *Sutterella* genus, which has been reported to be a potential probiotic present in pigeon “milk” that can improve the rate of growth and feed conversion ratio in chickens (Gillespie et al., 2012). The increase of the above mentioned bacterial taxa suggests that TM meal utilization (7.5% level of inclusion) may positively modulate the cecal microbiota of the free-range birds. On the contrary, the reduction of *Bacteroides* genus within the cecal community of the free-range chickens fed with TM of the present experiment may be considered a potential negative finding. Apart from being one of the most predominant genera found in chicken caecum (Callaway et al., 2009; Stanley et al., 2012; Wei et al., 2013), this taxon has been reported to be an important contributor to the intestinal health of the animals, because of its beneficial role for weight gain and growth performance (Chang et al., 2016) and the inhibition of *Clostridium perfringens* sporulation by its fermentation products (Wrigley, 2004). As a consequence, depletion of *Bacteroides* genus could be problematic for chickens, since it may predispose the animal gut to *Clostridium perfringens* infection and gastroenteritis (Wrigley, 2004). However, *Bacteroides* genus still outnumbered the other taxa in TM group of the present research and the birds fed with insects showed similar growth performance to those fed with control diet (see chapter 9.3.1).

9.3.6 Meat quality parameters

In the free-range chickens of the present experimental trial, breast pH falls in the range of “normal” meat (5.77 and 5.73 for C and TM groups, respectively). Differently, for values lower than 5.7 and higher than 6.2, breast broiler can be classified as PSE (pale, soft, and exudative) or DFD (dark, firm, and dry), respectively (Fletcher et al., 2000). The results of the current experiment are in contrast with Bovera et al. (2016), who reported a higher pH value in TM group compared to C diet. On the contrary, Cullere et al. (2016) reported a decrease in breast meat pH in broiler quails fed diets with increasing level of HI larvae meal and demonstrated that those differences could be ascribable to a different muscle glycogen content.

Meat colour is a very important quality parameter, since it is directly perceived by the consumer. In the present research, TM meal utilization did not influence colour parameters. To the best of PhD student’s knowledge, no published data are currently available comparing meat colour of free-range chickens fed with TM meals and other diets. In broiler chickens, Bovera et al. (2016) did not find any significant effects on raw and cooked color meat, as well as on skin, and they showed that meat from broilers fed with TM diet could be accepted without any problems by the consumers. A significant decrease for L* was reported by Pieterse et al. (2014) in breast muscle of broilers fed diets containing *Musca domestica* larvae meals compared to a fishmeal-based diet. Using HI larvae meal in

broiler quails, Cullere et al. (2016) observed that redness index (a^*) in the cranial and caudal part of the *Pectoralis major* muscle of broiler quails was significantly affected by the dietary treatment and showed its highest (1.13) and lowest (0.46) values for HI groups, corresponding to 10% and 15% HI inclusion levels, comparing to the control group (0.81).

As observed by previous studies, free-range system can reduce muscle fat content (Fanatico et al., 2007; Bogosavljevic-Boskovic et al., 2010; Chen et al., 2013). As a further, this variation was not observed in the present experimental trial. Regarding literature using insect meals, it must be pointed that the results of the current experiment are in agreement with those reported by Bovera et al. (2016). These authors did not find any significant effects on the chemical composition of meat obtained from right breast of broilers fed diets containing TM larvae meal during the growing period. The same results were mentioned by Cullere et al. (2017) and Schiavone et al. (2017b) in broiler quails and chickens fed diets with HI meal and fat, respectively. Ballitoc and Sun (2013), including different levels of TM meal (5, 10, 20 and 100 g/kg, respectively) in a standard commercial broiler diet, reported that the inclusion level of 10 g/kg TM showed the highest and lowest percentages of moisture in the thigh region and thigh area, respectively, as compared to the other groups. The same authors also reported that the same group showed a high percentage of protein in the breast portion of the meat. In parallel, the inclusion level of 100 g/kg TM showed the highest percentage of fat for thigh (6.30% vs 5.64 % of FM in our study) and breast (1.25% vs 0.50 % of FM in our study) muscles, respectively.

Even if the results of the present experimental trial showed that there were significant differences in some FA of the breast muscle between C and TM7.5 groups, the separate contributory effects of diet and rearing conditions could not be elucidated. However, in the current research, the FA profile of TM larvae meal did not reflect the FA profile of the analyzed muscles

Generally, free-range poultry had access to pasture that could lead to a higher content of PUFA, especially n-6, as demonstrated by Kralik et al. (2005), who detected that chickens reared outdoor had significantly higher portion of linoleic, arachidonic acid, and total PUFA n-6 acids than chickens that were kept indoors. In addition, chicken grown with the free-range rearing had access to grassland areas, so they could have eaten grass, insects, and worms that were not quantified in the present experiment. Furthermore, the current research was conducted in autumn, when insect availability is limited.

Rearing system can also influence the composition of n-3 and n-6 PUFA. Muriel et al. (2002) demonstrated that rearing animals outdoors significantly increased total n-3 and n-6 PUFA of meat. On the contrary, Castellini et al. (2002) reported that breast meat of free-range chickens contained lower n-3 PUFA, lower MUFA and higher fractions of SFA and PUFA in comparison with intensive production systems. Thus, the differences in the FA profile of breast meat in the present experimental trial cannot be related to the rearing conditions. Nevertheless, it appears that diet composition, especially TM meal inclusion, was the major determinant in modifying FA composition.

Indexes (AI, TI) correlating the different amount of some specific SFA, MUFA and PUFA of both n3 and n6 series were proposed to indicate the contribution of these FAs to the prevention or promotion of pathological phenomena. There were differences in AI and TI of the breast muscle from the birds fed with TM diet. Anyway, the obtained values were less or around 1.0, still considered to be healthy for human consumers.

9.4 EXPERIMENT 4

The present experimental trial is the first one which evaluated the effects of dietary defatted HI larvae meal inclusion on growth performance, haematochemical parameters, carcass traits, histological findings, gut health and meat quality and safety of broiler chickens.

9.4.1 Growth performance

Dietary HI meal inclusion positively influenced the growth performance of the birds in the present experimental trial, in terms of improved body weight and feed intake during the first period. The increased DFI observed in the starter period, which was accompanied by increased LW and ADG and unaffected FCR, was quite relevant. In fact, the starter period (from hatching to 10 days) is considered the most important in broiler production, since growth and development take place at an incredible rate in this period. Indeed, the chicks' weight quadruples, thus influencing the subsequent growth rate (Aviagen, 2014). Therefore, it appears that the inclusion of 10% meal in partial substitution of soybean meal is suitable for broiler chicken diets during the starter period. However, the feed efficiency was impaired in the animals fed HI diets during the growing and finisher periods.

There is currently a lack of information on the utilization of HI meal in broiler chickens. HI larvae and prepupae, grown on swine manure or kitchen waste, have been used satisfactorily as feed additives for young chicks (Hale, 1973). Elwert et al. (2010) demonstrated that increasing levels of defatted HI meal resulted in decreasing feed consumption and body weight of broiler chickens, but no significant effect on FCR was observed. The partial or full replacement of soybean cake with partially defatted HI meal in a diet for laying chickens did not affect their laying performance, or the feed efficiency, compared to organic standard diets for layers (Maurer et al., 2016). However, Marono et al. (2017) reported a low productive performance of laying hens when HI meal was used to totally replace soybean meal. In the same context, Cullere et al. (2016) showed that defatted HI meal could be introduced into the diet of growing broiler quails at 10 and 15% inclusion levels, to partially replace conventional soybean meal and soybean oil, with no negative effects on the productive performance or mortality. Al Qazzaz et al. (2016) reported similar feed intake, weight gain, haugh unit and hatchability in laying hens fed diets supplemented with 10 and 50 g/kg of HI meal. However, a

significant improvement was observed in the hens' daily egg production as a result of HI meal utilization. HI meal has been reported to improve the growth rate of chickens when used as a component of a complete diet (Oluokon, 2000). Loponte et al. (2017) reported that barbary partridges (*Alectoris barbara*) fed insect meals showed a higher live weight at 64 days than a group fed a soybean meal-based diet, when they studied the partial replacement of soybean meal with HI and TM insect meals. They reported that the higher live weight was not accompanied by a higher body weight gain for the TM and HI groups over the entire period of the trial, with a more favorable FCR appearing in the groups fed 25 and 50% of TM. The wide variability of the results obtained in the previous studies may be related to the nutritive value of the insect meal that was used, which can be influenced by the insect life stage (adult, larva or pupa), the insect rearing substrate, the defatting process (Sánchez-Muros et al., 2014) and also the growing period in which the chickens are fed.

9.4.2 Haematological and serum parameters

All of the blood parameters obtained in the present trial suggested that a partially defatted HI larvae meal does not affect the health status of animals. Furthermore, all the blood parameters obtained in the present study fell within the physiological ranges (Lumej et al., 2008). The H/L ratio has recently been used for the measurement of distress conditions in chickens, where an increased H/L ratio may indicate that the animals suffered from infections, inflammation or stress (De Marco et al., 2013; Pozzo et al., 2013; Salamano et al., 2010). The H/L ratio of chickens fed HI meal did not show any significant differences when compared with C group. No significant differences were also found for creatinine, thus implying that HI meal has similar effects on the kidney functions of the birds. The higher phosphorus serum concentrations observed in the HI birds could be related to the level of phosphorus in HI meal. The phosphorus content of HI larvae has been reported to be closely correlated with the phosphorus content of the provided substrate (Newton et al., 2005; Oonincx and de Boer, 2012). The results of the present research are in agreement with those of Schiavone et al. (2017b) and Li et al. (2016b), who showed that the use of HI larvae fat, in substitution of soybean oil, in broilers and juvenile Jian carp diets, respectively, had no negative effects on the blood traits of the animals, thus confirming the nutritional adequacy of these diets. Some studies concerning other insect species, such as *Tenebrio molitor* (Bovera et al., 2015), did not show any significant difference in the blood traits of the animals, thus confirming the findings of the current experimental trial. The main function of GPx, as part of the antioxidative defense system, is the removal or detoxification of hydrogen peroxides, thus serving as a major defense mechanism against oxidative damage. In this way, the enhancement of GPx activity can inhibit the production of reactive species. Many factors can disturb the equilibrium between the production of reactive species and the antioxidative defense system, such environmental toxicants as well as endogenous pathological metabolic changes (Jozanov-Stankov et al., 2009). The fat content of HI meal is composed of around 32% of saturated

fatty acids (SFA) (Schiavone et al., 2017b), and according to Simopoulos (1999), the saturated-to-unsaturated fat ratio should be taken into consideration due to its possible oxidative stress role.

9.4.3 Carcass characteristics

Feeding HI meal has been found to be effective in modifying the carcass traits of broiler chickens of the present experimental trial. The results of the relative carcass and organ weights showed a significant effect of HI meal inclusion, a result that could be positive for commercial purposes. On the basis of the results of the current research, the supplement of HI larvae meal at 10% led to the best outcomes. The results of the present experiment are consistent with the findings of Ballitoc and Sun (2013), who found improved slaughter, dressed carcass and eviscerated weights in broiler chickens fed TM diets with a 2% inclusion level. However, the results of the current research are not consistent with previous studies in broiler chickens and quails, in which no differences were observed for the relative organ weights as a result of house-fly maggots (*Musca domestica*, MD), yellow mealworm (*Tenebrio molitor*, TM) and HI meal (Cullere et al., 2016; Hwangbo et al., 2009) dietary inclusion. Loponte et al. (2017) also reported that the carcass traits of partridges (*Alectoris barbara*) were affected by insect meal utilization.

9.4.4 Histopathological findings

As already observed for the broiler chickens of the experimental trials 1 and 2, dietary HI meal inclusion did not affect the development or severity of the histopathological features of the birds of the present experiment, thus suggesting no negative influence on animal health. Similarly to the animals of the experimental trials 1 and 2, the broiler chickens of the current experiment showed different degrees of lymphoid system activation. As previously explained (see chapter 9.1.4), this result could be related to the stress occurrence in modern poultry rearing operations (Liles et al., 2015).

9.4.5 Gut mucosal morphology

Dietary HI meal inclusion has been found to partially affect the intestinal morphology of the broiler chickens in the current experimental trial. In particular, shorter villi, deeper crypts and reduced Vh/Cd were observed in the HI15 group when compared with the others. It is well known that the morphology of the small intestine, especially regarding the crypts and villi of the absorptive epithelium, plays a key role in the final phase of nutrient digestion and assimilation (Wang and Peng, 2008). The simultaneous observation of short villi and deep crypts is indicative of negative gut development. In

fact, larger crypt surfaces may be related to an increase in cell turn-over (Brunsgaard and Eggum, 1995), which can lead to a lower enterocyte maturity (Williams et al., 2008). An increase in cellular renewal also represents an increase in energy requirement for gut maintenance, with subsequent utilization of the nutrients for the functioning of the digestive tract instead of for growth (Williams et al., 2008). Furthermore, deeper crypts have been reported as being the host's intestinal attempt to compensate for normal sloughing or atrophy of the villi due to inflammation from pathogens and their toxins (Gao et al., 2008). Qaisrani et al. (2014) observed that low villus heights and greater crypt depths may lead to poor digestion and less absorption of nutrients and, as a consequence, a poor performance of the animals, and they attributed this to the modifications of the protein source and dietary structure. The altered morphometric indices observed in male broilers fed a 15% level of HI meal inclusion could be related to the higher level of insects in this diet, thus also suggesting that lower levels may be preferable. A similar consideration can also be made in relation to the growth performance observed in the present study. The LW and DWG also increased linearly and/or quadratically till 10% of HI meal inclusion, and decreased (often dramatically) till the inclusion of 15% of insects. The overall FCR also increased linearly with increasing levels of HI meal, reaching its maximum for the inclusion of 15% of insects. Furthermore, the alterations of the gut morphology can also explain the observed growth performance. Indeed, considering that the rapid growth of chickens depends directly on the morphological and functional integrity of the digestive tract (Wang et al., 2008), the relationship between the negative gut morphometric indices and the deterioration of the growth performance seems logical, as already suggested for the male broiler chickens of the experimental trial 1 (see chapter 9.1.5).

9.4.6 Gut mucin composition

The broiler chickens fed with 10 and 15% levels of HI meal inclusion of the current experimental trial showed lower mucin staining intensity than those fed with C and HI5 diets in the intestinal villi. As already mentioned before, Forder et al. (2007) pointed out that bacteria may influence the mucin production. In particular, bacteria such as *Helicobacter pylori* possess the enzymatic ability to disrupt the oligomeric structure of mucin, enabling the pathogen to move freely in the mucus layer, assisted by its highly active flagellum and its ability to down-regulate mucin synthesis (Deplancke and Gaskins, 2001). Interestingly, *Helicobacter* genus was one of the characteristic OUTs of the cecal microbiota of the birds fed with 15% level of HI meal inclusion of the present research (see chapter 9.4.7). Therefore, it is reasonable to hypothesize a direct relationship between the reduced gut mucin production and identified bacterial population. Furthermore, as already hypothesized for the male broiler chickens of the experiment 1, the utilization of HI meal at low inclusion rates (i.e., 5%) may be preferable to preserve the protective properties of mucin glycoproteins in order to optimize the digestive process and to prevent the enteric infections.

Independently of HI meal utilization, the intestinal crypts of the birds of the current trial showed lower acidic sulfated mucin staining intensity. Similarly to what reported for the male broiler chickens of the experimental trial 1, greater acidic sialylated mucin staining intensity than acidic sulfated was observed in the intestinal villi. Despite the limited information currently available about mucin dynamics in crypts and villi, the physiological relevance of the different mucin subtypes has, however, been suggested. In particular, a high degree of sulfation within the acidic mucins is characteristics for immature goblet cells (Turck et al., 1993). Therefore, the assessment of mucin types in the current experiment is indicative of overall mature and health guts with well-developed mucin secretory architecture.

Independently of dietary HI meal inclusion, the intestinal crypts and villi of the broiler chickens of the present research showed greater mucin staining intensity in the ileum compared with the other gut segments. This is similar to what observed for the chickens of the previous experimental trials and is consistent with previous findings in chickens (Uni et al., 2003; Forder et al., 2007), which described different mucin dynamics depending on the gut segment considered (see chapter 9.1.6).

As already reported for the chickens of the previous experimental trials, the intestinal crypts of either C or HI birds of the current experiment showed greater mucin staining intensity in the base compared with the other fragments. This is in agreement with previous studies (Uni et al., 1998; Tsirtsikos et al., 2012a) and it is related to the physiological goblet cells proliferation and maturation process (see chapter 9.1.6).

9.4.7 Cecal microbiota characterization

In the broiler chickens of the present experimental trial, cecal microbiota was mainly colonized by the phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria*, as previously reported in normal chickens (Wise and Siragusa, 2007; Tillman et al., 2011; Wei et al., 2013; Oakley et al., 2014). However, the predominance of the phylum *Proteobacteria* (with particularly high levels in HI15 birds) over *Bacteroidetes* is in contrast with these studies and difficult to explain. High numbers of *Proteobacteria* members are generally considered a sign of bad intestinal health and are associated with gastrointestinal health conditions such as chronic dysbiosis (Shin et al., 2015) and inflammatory bowel disease (Mukhopadhyaya et al., 2012). However, the birds of the current research showed no signs of gut inflammation. Another interesting aspect to underline is that the cecal microbiota of the broiler chickens of the current research showed lower OTUs richness and alpha diversity when fed with 15% level of HI meal inclusion compared to the other diets. This represents a potential negative finding, since high levels of diversity have been reported to help maintain the stability of intestinal microbiota after environmental stress (Xue et al., 2015) and determine the colonization resistance against invading pathogens (Kuhn et al., 1993).

Differently from the previous experimental trials, each group of the current research showed a specific signature at genus level in the cecal microbiota. Firstly, the broiler chickens fed with C diet showed the Lachnospiraceae family as characteristic OUT in their cecal microbiota. It is well known that Lachnospiraceae, along with Ruminococcaceae, is a typical butyrate-producing family (Li et al., 2017). Butyrate has various positive properties, since it nourishes the enterocytes, increases intestinal mucin production (Jung et al., 2015), improves tight-junction integrity (Peng et al., 2009), takes part in cellular differentiation and proliferation within the intestinal mucosa (Rinttila and Apajalahti, 2013) and can reduce an inflammatory response as an anti-inflammatory effector (Furusawa et al., 2013).

Secondly, the birds fed with HI5 diet showed *L-Ruminococcus*, *Faecalibacterium*, *Blautia* and *Clostridium* as characteristic OTUs of their cecal microbiota. Similarly to the already described members of Lachnospiraceae family, *Faecalibacterium* and *Clostridium* genera encompass members capable of producing butyric acid (Pryde et al., 2002; Louis and Flint, 2009). Furthermore, *Blautia* is a new genus belonging to the Ruminococcaceae family that can produce short chain fatty acids through glucose metabolism and digest cellulose in food (Liu et al., 2008). As already mentioned before, short chain fatty acids represent a remarkable source of energy for enterocytes (Sunkara et al., 2012) and are able to suppress gut pathogens (Rehman et al., 2007). Another aspect to recall is that *Clostridium* is one of the most predominant genera identified in chicken caecum (Gong et al., 2007; Stanley et al., 2012; Danzeisen et al., 2011; Wei et al., 2013; Costa et al., 2017).

Thirdly, the broiler chickens fed with 10% level of HI meal inclusion showed *Lactobacillus* and *Ruminococcus* as characteristic OTUs of their cecal microbiota. The positive effects of Ruminococcaceae family (of which *Ruminococcus* genus is a member) have already been described. In parallel, *Ruminococcus* and *Lactobacillus* have been reported as two of the most predominant genera found in chicken caecum (Gong et al., 2007; Stanley et al., 2012; Danzeisen et al., 2011; Wei et al., 2013; Costa et al., 2017). As a typical probiotic bacterium, *Lactobacillus* also promotes the homeostasis of immune cells and intestinal health of the host (Van Tassell and Miller, 2011; Ren et al., 2016). Furthermore, the lactate produced by *Lactobacillus* species can be converted to short chain fatty acids (Elferink et al., 2001; Duncan et al., 2004; Bjerrum et al., 2006).

Finally, the birds fed with HI15 diet showed *Bacteroides*, *Roseburia* and *Helicobacter* as characteristic OTUs of their cecal microbiota. Apart from being one of the most predominant genera found in chicken caecum (Callaway et al., 2009; Stanley et al., 2012; Wei et al., 2013), *Bacteroides* taxon has been reported to be an important contributor to the intestinal health of the animals, because of its beneficial role for weight gain and growth performance (Chang et al., 2016) and the inhibition of *Clostridium perfringens* sporulation by its fermentation products (Wrigley, 2004). Furthermore, *Roseburia* has been reported to be a butyrate-producing genus (Scott et al., 2011). However, high abundance of *Helicobacter* genus can be considered a potential negative finding. Indeed, despite its potential to remove hydrogen that can benefit the other members of the microbial community and the host's ability to recover energy from food (Sergeant et al., 2014), it harbors some specific enterohepatic *Helicobacter* species (such as *Helicobacter pullorum*) (Stanley et al., 1994).

Furthermore, bacteria such as *Helicobacter pylori* (which were detected in the HI15 broilers of the current research) possess the enzymatic ability to disrupt the oligomeric structure of mucin, enabling the pathogen to move freely in the mucus layer, assisted by its highly active flagellum and its ability to down-regulate mucin synthesis (Deplancke and Gaskins, 2001). Interestingly, the animals fed with 10 and 15% levels of HI meal inclusion (especially the 15%) showed reduced mucin production in the intestinal villi (see chapter 9.2.6), thus suggesting a direct influence of microbiota on mucin dynamics. The identification of *L-Ruminococcus*, *Faecalibacterium*, *Blautia*, *Clostridium*, *Ruminococcus*, *Lactobacillus*, *Bacteroides* and *Roseburia* as characteristic bacterial taxa of HI animals suggests that HI meal utilization may positively modulate the cecal microbiota of birds. However, the reduced microbial diversity and the high prevalence of *Helicobacter* genus identified in the birds fed with 15% level of insect meal inclusion (with subsequent negative modulation of gut mucin dynamics) suggests that lower HI levels (in particular the 5%) could be preferable. This can also be confirmed by gut mucosal morphology findings, since the birds fed with 15% level of HI meal inclusion showed the worst intestinal development in terms of shorter villi, deeper crypts and reduced villus height to crypt depth ratio (see chapter 9.4.5). Interestingly, the broilers fed with HI15 diet also displayed the worst growth performance in terms of highest feed conversion ratios (see chapter 9.4.1). Even if a direct cause-effect relationship between diversity and composition of cecal microbiota and bird performance has not yet been confirmed, the results of gut morphology, mucin composition and microbiota taken together suggest a potential influence of the intestinal features on the growth of animals (as already hypothesized for the male broiler chickens of the experimental trial 1).

9.4.8 Meat quality parameters

Raw meat quality can be described by the pH, color, water holding capacity and tenderness, all of which can be affected by several dietary and environmental variables (Cavani et al., 2009). The color of a raw meat product is an important determinant in the selection of meat by the consumer. Consumers will often reject products in which the color varies from the expected normal appearance and subsequently this parameter is often used to determine the economic value of food (Wideman et al., 2016). The results of the color characteristics of breast muscle from chickens fed a diet with different levels of HI meal of the present experimental trial showed that the redness (a^*) index was higher in the HI15 group, while the HI5 and HI10 groups showed intermediate values in comparison with the control group. These findings may be related to the possible accumulation of insect meal pigments in the intramuscular fat. On the other hand, the yellowness value (b^*) decreased in a linear fashion. This result may be attributed to the decrease of corn gluten in the diets, as the HI meal inclusion level increased. The findings of the current experiment are consistent with Cullere et al. (2016), who showed that meat redness (a^*) was affected significantly by the increasing inclusion level of HI meal in quail diets. However, Leiber et al. (2017) did not find any significant effects of HI meal on

the color of broiler meat. The use of other insect meals in broiler diets has also been associated with a significant decrease in lightness (L^*) in the breast muscle of birds fed diets containing MD larva meals, when compared to a fish meal-based diet (Pieterse et al., 2014). Bovera et al. (2016) did not find any significant influence on the color of raw and cooked meat or on the skin of broiler chickens and they demonstrated that the meat from broilers fed TM meal could be accepted by consumers. As far as the chemical composition of the breast muscle is concerned, an orthogonal polynomial contrast test revealed that the moisture and protein content decreased and increased, respectively, with increasing HI meal inclusion levels. Intramuscular fat is very important, from the sensory point of view, since it is a source of many aromatic substances that affect the taste of meat (Suchý et al., 2002). In the present experimental trial, the dietary HI meal supplementation increased the breast meat fat content up to 10% of HI meal. As a result, HI15 showed a 0.19% greater lipid content than C diet. The improvement of the intramuscular lipid content indicates a good availability and storage of nutrients. Such findings are in contrast with Cullere et al. (2017) and Schiavone et al. (2017b), who reported no significant effects on meat chemical composition of broiler quails and chickens fed diets with HI meal and fat, respectively. As far as the literature related to the use of insect meals is concerned, it should be pointed out that the results of the current research are in disagreement with those reported by Bovera et al. (2016), who instead reported a significant influence on the breast meat composition of broilers fed diets containing TM meal during the growing period. Ballitoc and Sun (2013), who included different levels of TM meal (5, 10, 20 and 100 g/kg, respectively) in a standard commercial broiler diet, showed a significantly higher percentage of moisture in the thigh muscle of HI10 birds compared to the other groups, associated with a higher protein content in the breast meat. Moreover, an inclusion level of 100 g/kg of TM showed the highest percentage of fat in the thigh (6.30%) and breast (1.25%) muscles, respectively. The same authors also observed a slight increase of the protein content in the TM groups compared to the C diet. However, these differences could be ascribed to the rearing substrates of the HI larvae.

The FA content of broiler meat basically depends on the dietary fat profile. Several factors (i.e., genotype, sex, housing system, age, slaughter weight) are also capable of affecting the FA composition of meat, the diet component being considered as the major influencing factor in monogastric animals (Rymer and Givens, 2005; Schiavone et al., 2010; Schiavone et al., 2007). Consequently, as expected, the dietary inclusion of a defatted HI meal greatly influenced the FA profile of the broiler breast meat of the present experimental trial. Dietary HI meal inclusion induced significant modifications in the proportions of most of the FAs in the breast meat. The lauric and myristic acid contents increased with increasing levels of dietary HI meal inclusion to a lesser extent, with the same increase being also noticed for C16:0. However, the total SFA (C18:0) remained constant, while C24:0 reduced linearly as the HI inclusion rate increased. The results of the current research are in agreement with Cullere et al. (2017) and Renna et al. (2017) pertaining to broiler quails and trout fed defatted HI meal. In juvenile Jian carp (*Cyprinus carpio* var. Jian) fed HI oil, Li et al. (2016) found relatively lower levels of lauric acid in the muscles of HI-fed trout than the respective

lauric acid dietary levels, thus suggesting a ready utilization of this FA for energy production. An increasing percentage of MUFA, mainly due to the higher content of C18:1 n-7, was observed as a result of increasing dietary HI meal inclusion levels. On the contrary, PUFA significantly decreased for greater decreases in C18:2 n-6. HI meal was found to be relatively scarce in PUFA, which were represented almost entirely by C18:2 n-6 and C18:3 n-3. Such a composition lowered the contents of the n-6 and the n-3 PUFA fractions as the dietary HI meal level increased. Despite these remarkable changes in the breast meat FAs, the Σ n-6/ Σ n-3 ratio remained unaffected.

Breast meat lipids are mainly composed of triacylglycerol and phospholipids, the latter being particularly rich in very long chain n-3 PUFA (EPA and DHA), which are well known for their high biological efficiency in the organism and their beneficial effects on human health (Rymer and Givens, 2005). In the present experimental trial, a significant effect was only observed for EPA and DHA with decreasing levels of HI meal to 10%. These findings are in agreement with Cullere et al. (2017), who found significant reductions of DHA in the breast meat of Japanese quail fed increasing levels of HI meal. Renna et al. (2017) found lower contents of EPA and DHA in the fillet muscles of trout fed 40% of HI than in trout fed other diets, while EPA was reduced when the lowest level of HI meal was used. Regarding the nutritional and human health-related indexes, the Σ PUFA/ Σ SFA, Σ n-6/ Σ n-3 ratio as well as AI and TI were significantly higher in the broilers fed with HI diets than C. All the indexes deteriorated as the dietary HI meal inclusion level increased.

The present research indicates that dietary defatted HI meal inclusion in broiler chicken leads to modifications of the breast meat lipid profile, with a higher MUFA and n-6/n-3 ratio and lower PUFAs, particularly the very long chain n-3 FAs (EPA and DHA). In order to balance these potential negative effects of a diet rich in HI meal and to overcome the negative effects on the FA profile of the breast meat, a modulation of the larvae rearing substrate appears to be necessary to obtain an improved FA profile of HI, as already pointed out in previous studies (Tschirner and Simon, 2015; Spranghers et al., 2017).

9.4.9 Microbiological findings

Safety issues are of great concern when insects are intended for use in feeds. One of the potential risks associated with the use of insect protein is represented by microbiological threats (Charlton et al., 2015).

In the current experimental trial, *Salmonella* spp. was not detected in neck skin samples of both birds fed with C and HI diets, thus being in compliance with EU norms (EC, 2005). Similarly, *Salmonella* spp. and *Campylobacter* spp. were not identified in breast meat of either broiler chickens fed with C or HI diets, thus being also in compliance with EU norms (EC, 2005). The conformity to process hygiene and food safety criteria represents a quite relevant finding in terms of food safety.

9.4.10 Heavy metals and arsenic content

Among the chemical hazards related to insect meal utilization, such heavy metals as cadmium, lead and mercury as well as metalloid arsenic should be taken into account (Charlton et al., 2015).

In the current experimental trial, the concentrations of heavy metals and arsenic in both HI meal and breast meat of the birds remained below the EU limits suggested for feed materials (EC, 2002) and foodstuffs (EC, 2006). HI larvae have been reported to accumulate cadmium (Charlton et al., 2015; Van der Fels-Klerx et al., 2016; Biancarosa et al., 2017), lead (Van der Fels-Klerx et al., 2016; Biancarosa et al., 2017; Purschke et al., 2017), mercury (Biancarosa et al., 2017) and arsenic (Charlton et al., 2015; Van der Fels-Klerx et al., 2016; Biancarosa et al., 2017) when reared on contaminated substrates. The results obtained in the present research suggest that HI meal was fed with a feeding media that did not contain heavy metal or arsenic levels that exceeded the maximum allowable limits. The cadmium and lead concentrations in the breast meat also remained below the EU limits, thus representing a quite relevant result in terms of food safety.

The breast meat of the birds fed with HI meal showed numerically lower iron, nickel, copper and selenium contents than that of the animals fed C diet. However, since these results were obtained on pools and not on single birds, a statistical analysis could not be performed and no significant considerations can be drawn in relation to these data.

10 CONCLUSIONS

The present PhD project evaluated the effects of dietary *Tenebrio molitor* (TM) and *Hermetia illucens* (HI) meal utilization on animal health and performance, gut health and meat quality and safety of different chicken strains. In particular, the present PhD project was focused on the development of a standardized and multidisciplinary *post mortem* approach to assess gut health when dietary modifications occur in poultry, in order to evaluate a potential relationship with animal performance and quality and safety of the obtained meat products.

Gut health assessment in the birds of the present PhD project consisted of the parallel evaluation of gut mucosal morphology, gut mucin composition and gut microbiota. All these investigations give different, specific and complementary information about the health status of intestinal environment, thus underlying the importance of a *post mortem* multidisciplinary approach.

Increasing levels of dietary TM and HI meal inclusion (i.e., 15%) affected the gut mucosal morphology of the male broiler chickens of the experiments 1 and 4, respectively, in terms of shorter villi, deeper crypts and reduced Vh/Cd. The simultaneous observation of short villi, deep crypts and low Vh/Cd is indicative of negative gut development, since these morphological features have been reported to cause poor digestion and less absorption of nutrients and, as a consequence, poor performance of the animals. These altered morphometric indices could be related to the higher level of insects of these diets, thus also suggesting that lower levels may be preferable. Differently, the female broiler chickens of the experiment 2 showed unaffected gut morphometric indices when fed with TM meal inclusion (even when 15% level was included), thus suggesting no negative influence of insects on gut health, development and functionality. These findings may be related to the different sex or length of the production cycle, even if further investigations are needed to confirm this hypothesis. Similarly, the free-range chickens (experiment 3) also showed unaffected gut mucosal morphology when fed with yellow mealworm meal (7.5% level of inclusion). This result could reflect the poultry's innate feeding habit of naturally consuming insects when reared in free-range systems. However, independently of dietary TM and HI meal inclusion, all the animals showed a proximodistal decreasing gradient of morphometric indices from duodenum to ileum. This represents a relevant finding in terms of gut health, since insect meal utilization allowed the preservation of the physiological gut development and absorption processes.

Gut mucin composition evaluation revealed greater mucin staining intensity in the intestinal crypts or villi of the broiler chickens fed with 5% level of insect meal inclusion when compared with 10 and 15% (experiments 1, 2 and 4). Furthermore, the broiler chickens fed with 10% level of TM meal inclusion (experiment 2) and 10 and 15% levels of HI meal inclusion (experiment 4) showed lower mucin staining intensity than those fed with C diets in the intestinal villi. Independently of diet, a potential influence of microbiota on mucin synthesis needs to be taken into account. Indeed, bacteria such as *Helicobacter pylori* are able to produce mucin-degrading enzymes. Interestingly, *Helicobacter* genus

reached the highest percentage in the females fed with 10% inclusion level of TM meal and was one of the characteristic OUTs of the cecal microbiota of the birds fed with 15% level of HI meal inclusion. As a consequence, it seems reasonable to hypothesize a direct relationship between the reduced gut mucin production and identified bacterial population. However, independently of microbiota, mucins constitute a digestion- and absorption-assisting medium and act as a substrate for the resident flora and by aggregation facilitate the removal of pathogenic bacteria. Therefore, the utilization of insect meal at low inclusion rates (i.e., 5%) could be preferable to preserve the protective properties of mucin glycoproteins in order to optimize the digestive process and to prevent the enteric infections. Differently from broilers, the free-range chickens (experiment 3) showed unaffected gut mucin composition when fed with TM meal. As already suggested for gut mucosal morphology, this result could reflect the poultry's innate feeding habit of naturally consuming insects when reared in free-range systems. Another important aspect to underline is that, independently of dietary TM and HI meal inclusion, all the animals preserved the physiological gut mucins dynamics (identification of greater mucins staining in the crypt base, lower mucins staining in the caecal crypts, higher mucins staining along the duodenal-ileal axis and greater neutral and acidic sialylated mucins staining), thus suggesting that insect meal allows the maintenance of overall mature and health guts with well-developed mucin secretory architecture.

The global diversity of cecal 16S rRNA sequences identified in all the animals is in agreement with literature findings about physiological chicken microbiota, in terms of both bacterial phyla (overall predominance of *Bacteroidetes*, *Firmicutes* and *Proteobacteria*) and genera (overall predominance of *Clostridium* / members of *Clostridiales*, *Ruminococcus* / members of *Ruminococcaceae* and *Bacteroides*). Interestingly, high percentages of *Alistipes* (not so frequently identified in normal chicken microbiota) and *Copro bacter* (first description in poultry microbiota) genera were also observed in the male and female broilers of the experiments 1 and 2, respectively. Furthermore, *Helicobacter* genus was identified in the female and male broilers of the experiments 2 and 4, respectively, as one of the most predominant cecal members. Depending on the specific experiment, a variable modulation of the cecal microbiota by insect meal inclusion was observed. Overall positive modulation of cecal microbiota by dietary TM meal inclusion was observed in the free-range chickens of the experiment 3, in terms of preservation of the physiological microbial population (increased abundance of *Clostridium* and *Ruminococcus* genera) and selection of potential beneficial bacteria (increased abundance of butyrate-producing genera – *Oscillospira* and *Copro coccus* – and growth performance-ameliorating bacteria – *Sutterella* genus). Differently, the female and male broilers of the experiments 2 and 4 showed only a partial positive modulation of their cecal microbiota by dietary TM and HI meal inclusion, respectively. Indeed, the positive findings were related to preservation of the physiological microbial population (overall increased abundance of *Clostridium*, *Ruminococcus*, *L-Ruminococcus*, *Bacteroides* and *Lactobacillus* genera) and selection of potential beneficial bacteria (overall increased abundance of butyrate-producing genera – *Faecalibacterium*, *Blautia* and *Roseburia* – growth performance-ameliorating bacteria – *Sutterella* and *Bacteroides* genera – and gut

beneficial bacteria – *Alistipes* genus). However, the potential negative modulation was represented by the decreased and increased abundances, respectively, of *Ruminococcus* (female broilers fed with TM meal of the experiment 2) and *Helicobacter* (male broilers fed with 15% level of HI meal inclusion of the experiment 4) genera. Finally, overall negative modulation of the cecal microbiota by dietary TM meal inclusion was observed in the male broiler chickens of the experiment 1 (especially in the birds fed with 10 and 15% levels of insect meal inclusion), in terms of partial alteration of the physiological microbial population (decreased abundance of *Clostridium*, *Ruminococcus* and *L-Ruminococcus* genera) and positive bacteria (decreased abundance of butyrate-producing *Coprococcus* genus). Another important aspect to underline is that, even if less evident when compared to gut mucosal morphology and mucin composition, the negative modulation of gut microbiota was particularly evident with high levels (especially the 15%) of dietary TM and HI inclusion. Therefore, also the microbial findings seem to suggest that low inclusion rates of insect meal could be preferable for poultry diets. Another interesting, but difficult to define and explain, aspect to underline is the evident change in phyla composition in relation to the different experimental trials. In the broiler chickens of the experiments 1 and 2, as well as the free-range chickens of the experiment 3, the dominant bacterial phyla, from high to low, were *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. Differently, the broiler chickens of the experimental trial 4 showed a clear predominance of *Firmicutes* and *Proteobacteria* phyla over *Bacteroidetes*. Apart from the free-range chickens, whose phyla changes can be explained by the different genetics and rearing conditions, the findings observed in broiler chickens appear to be particularly contrasting among each other. The only aspect which differed in all the three experiments was the slaughtering age (53 days for the experimental trial 1, 40 days for the experimental trial 2 and 35 days for the experimental trial 4). It has been identified that age has a large effect on the chicken intestinal microbiota (Kers et al., 2018). Furthermore, the role of the environmental factors (i.e., biosecurity level, housing, litter, feed access and climate) can not be excluded (Kers et al., 2018).

In regards to the animal performance, the broiler chickens of the experiments 1, 2 and 4 showed improved body weight and feed intake with increasing levels of dietary TM and HI meal inclusion. These positive findings were considered suggestive of increased feed palatability in relation to the addition of insect meals, since insects are naturally consumed by wild birds and free-range poultry. However, the increase of the body weight and feed intake resulted in an overall impairment of the feed efficiency in the birds fed TM and HI diets (especially the male broilers). This detrimental effect was attributed to the chitin contained in the exoskeleton of the insects, which has been reported to be difficult to digest by domestic poultry and may further negatively influence the apparent digestibility coefficient of the total tract of nutrients. Differently from broilers, the free-range chickens of the experiment 3 showed unaffected growth performance when fed with TM diet (7.5% level of inclusion). As already suggested for gut mucosal morphology and mucin composition findings, this result could reflect the poultry's innate feeding habit of naturally consuming insects when reared in free-range

systems. However, since gut health is of vital importance to animal performance, all these results need to be contextualized with those obtained during the *post mortem* assessment of gut health aspects. Indeed, the male broilers fed with 15% level of dietary TM and HI meal inclusion (experiments 1 and 4, respectively) showed the worst gut mucosal morphology in terms of shorter villi, deeper crypts and reduced Vh/Cd, along with reduced gut mucin staining intensity (observed in the HI birds) and partial negative modulation of the cecal microbiota (observed in both TM and HI animals). Furthermore, even if much less evident when compared to the males, the female broilers fed with 10% level of dietary TM meal inclusion (experiment 2) showed reduced gut mucin staining intensity and partial negative modulation of the cecal microbiota (particularly high percentage of *Helicobacter* genus). Finally, the free-range chickens fed with TM meal (experiment 3) showed unaffected gut mucosal morphology, mucin composition and microbiota. Even if a clear cause-effect relationship between diversity and composition of cecal microbiota and bird performance has not yet been confirmed, the results of gut morphology, mucin composition and microbiota taken together suggest a potential influence of the intestinal features on the growth of animals.

In relation to meat quality and safety, overall technological properties and proximate composition of breast and thigh meat were unaffected by dietary TM and HI meal inclusion in both free-range (experiment 3) and male broiler (experiment 4) chickens. However, contrasting results were obtained in regards to nutritional quality and health properties of fat: indeed, while insect meal utilization positively affected myristic and α -linolenic acids concentrations, total n-3 fatty acids amounts, the $\Sigma n-6/\Sigma n-3$ fatty acids ratio and the atherogenicity and thrombogenicity indexes in the free-range chickens, a reduction in the PUFA content was observed in the broiler chickens fed with HI meal. These differences, which can be attributed to the different fat profile of the two insect used, may be smoothed by the modulation of the larvae rearing substrate. The most important aspect to consider in a context of food quality and safety is that dietary HI meal inclusion showed no negative effects on both microbiological findings (absence of *Salmonella* and *Campylobacter* spp.) and heavy metals content (identification of mercury, cadmium, lead and arsenic levels in compliance with norms) of meat. These findings represent the first data about meat safety aspects in poultry fed with insects. Furthermore, the absence of pathogens in meat reflects the absence of pathogens in the intestinal environment.

Another aspect to underline is that dietary TM and HI meal inclusion did not negatively affect the health status of both broiler and free-range chickens of the four experimental trials. Indeed, the mortality rates were neglectable and not influenced by insect meal utilization. The blood and serum parameters also fell within the physiological ranges reported for birds and were unaffected by dietary TM and HI meal inclusion. Furthermore, insect meal utilization did not influence either the development or severity of the histopathological alterations. The broiler chickens of the experiments 1, 2 and 4 showed different degrees of lymphoid system activation in absence of recognizable

diseases in the other organs (intestine included), which were attributed to the stress occurrence in modern poultry rearing operations. A potential role of TM and HI meals in the development of these alterations seems unlikely as they were also found in the animals fed with the control diet, their severity was unaffected, and the percentage of their occurrence was heterogeneous and quite similar among the dietary treatments. However, the greater percentage of normal and moderately affected organs in both birds fed with control and insect diets may suggest the presence of tolerable stress levels. This hypothesis could also be supported by the identification of the overall positive health and welfare status of the broilers. Differently from broilers, the free-range chickens of the experiment 3 showed different degrees of lymphoid system activation along with chronic inflammation of the intestinal segments. The most reliable hypothesis is that the lymphoid system activation represents a consequence of gut inflammation, even if the outdoor access have been reported to determine a stimulation of the lymphoid organ activities in free-range birds. The glandular stomach alterations (chronic inflammation with lymphoid tissue activation and epithelial squamous metaplasia) were considered para-physiological in relation to the free-range farming system, where outdoor access may lead to less control of animal feeding and subsequent possible ingestion of foreign bodies.

As a final conclusion of the present PhD project, TM and HI meal utilization does not negatively affect animal health and safety of the obtained meat products in chickens. However, high levels of inclusion (especially the 15%) can lead to negative modulation of gut health aspects (mucosal morphology, mucin composition and microbiota) and animal performance (feed efficiency), thus suggesting that lower levels (in particularly the 5%) may be preferable. Anyway, the identification of physiological cecal community and gut morphological development and mucin dynamics in all the animals (observed independently of insect meal utilization) makes insects particularly promising as alternative protein source in poultry diet. Furthermore, the parallel evaluation of both animal performance and gut health aspects also confirmed the strict relationship existing between the growth of chickens and morphological and functional integrity of the digestive tract, thus suggesting that gut health assessment by a *post mortem* multidisciplinary approach (herein developed and standardized) appears to be fundamental.

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12 RESEARCH PRODUCTS

12.1 Articles

- Bellino C., Iussich S., **Biasato I.**, Peletto S., Caruso C., Gianella P., Cagnasso A., D'Angelo A.: "Potential pathogenetic role of bovine herpesvirus 4 (BoHV-4) in two dairy cows with the dermatitis, pyrexia and hemorrhagic syndrome (DPHS)." - J Clin Microbiol 2015 Aug;53(8):2763-7.
- **Biasato I.**, Francescone L., La Rosa G., Tursi M.: "Anatomopathological staging of feline hypertrophic cardiomyopathy through quantitative evaluation based on morphometric and histopathological data." - Res Vet Sci 2015 Oct;102:136-141.
- **Biasato I.**, De Marco M., Rotolo L., Renna M., Lussiana C., Dabbou S., Capucchio M.T., Biasibetti E., Costa P., Gai F., Pozzo L., Dezzutto D., Bergagna S., Martinez S., Tarantola M., Gasco L., Schiavone A.: "Effects of dietary *Tenebrio molitor* meal inclusion in free-range chickens." - J Anim Physiol Anim Nutr (Berl) 2016 Mar;100:1104-1112.
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- **Biasato I.**, Tursi M., Longato E., Zanet S., Capucchio M.T.: "Pulmonary artery dissection causing haemothorax in a cat: potential role of *Dirofilaria immitis* infection and literature review." - J Vet Cardiol 2017 Feb;19:82-87.
- **Biasato I.**, Biasibetti E., Gili S., Bruatto G., Tarducci A., Guarda F., Capucchio M.T.: "Right atrial appendage aneurysms in veal calves and beef cattle: anatomopathological investigations and aetiopathogenetic hypotheses." - J Vet Cardiol 2017 Feb;19:68-73.
- Schiavone A., Cullere M., De Marco M., Meneguz M., **Biasato I.**, Bergagna S., Dezzutto D., Gai F., Dabbou S., Gasco L., Dalle Zotte A.: "Partial or total replacement of soybean oil by black soldier fly larvae (*Hermetia illucens* L.) fat in broiler diets: effect on growth performances, feed-choice, blood traits, carcass characteristics and meat quality." - Ital J Anim Sci 2017 Feb;16:93-100.

- **Biasato I.**, Lanteri G., Guarda F., Capucchio M.T., Marino F., Briguglio G., Macrì F.: "Unusual Combination of Multiple Vascular Anomalies in a German Shepherd Puppy with Megaoesophagus." - Anat Histol Embryol 2017 Apr;46:216-219.
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- Candini D., **Biasato I.**, Dell'Armeline Rocha P.R., Grego E., Capucchio M.T., Vercelli C.: "How Behavioral Changes Can Indicate Serious Cerebral Pathology: A Case Report of Concomitant Olfactory Neuroblastoma and Distemper Virus Encephalitis in a Swiss Shepherd Dog." - Vet Sci 2017;4:42.
- **Biasato I.**, Gasco L., De Marco M., Renna M., Rotolo L., Dabbou S., Capucchio M.T., Biasibetti E., Tarantola M., Sterpone L., Cavallarin L., Gai F., Pozzo L., Bergagna S., Dezzutto D., Zoccarato I., Schiavone A.: "Effects of yellow mealworm larvae (*Tenebrio molitor*) inclusion in diets for female broiler chickens: implications for animal health and gut histology." - J Anim Feed Sci Technol 2017 Dec;234:253-263.
- **Biasato I.**, Gasco L., De Marco M., Renna M., Rotolo L., Dabbou S., Capucchio M.T., Biasibetti E., Tarantola M., Bianchi C., Cavallarin L., Gai F., Pozzo L., Dezzutto D., Bergagna S., Schiavone A.: "Yellow mealworm larvae (*Tenebrio molitor*) inclusion in diets for male broiler chickens: effects on growth performance, gut morphology and histological findings." - Poult Sci 2018 Feb 1;97(2):540-548.

12.2 Abstracts and Posters

- **Biasato I.**, Biasibetti E., De Marco M., Rotolo L., Gasco L., Schiavone A., Capucchio M.T.: "Dietary insect meal inclusion in chickens: preliminary results about anatomopathological investigations." - LXIX Convention S.I.S.Vet (Perugia, 15-16-17th of June, 2015).
- **Biasato I.**, Capucchio M.T., Biasibetti E., Bruatto G., Cenacchi G., Guarda F.: "Coronary arteriosclerosis in veal calves and beef cattle: potential relation to housing conditions." - LXIX Convention S.I.S.Vet (Perugia, 15-16-17th of June, 2015).

- Gattino F., Iussich S., **Biasato I.**, Gola C., Maniscalco L., De Las Mulas JM., Millàn Y., Buracco P., De Maria R.: "Expression of PDGFRs receptors in canine mammary carcinomas: pathological and clinical implications." - LXIX Convention S.I.S.Vet (Perugia, 15-16-17th of June, 2015).
- Gattino F., Iussich S., **Biasato I.**, Gola C., Maniscalco L., De Las Mulas JM., Millàn Y., Buracco P., De Maria R.: "Expression of PDGFRs receptors in canine mammary carcinomas: pathological and clinical implications." - ESVONC Congress Krakow (Cracovia, 28-29-30th of May, 2015).
- Schiavone A., De Marco M., **Biasato I.**, Gasco L.: "Utilization of insects as feed in the production of broiler chickens." - Convegno: "Insetti edibili: aspetti tecnico-scientifici e normativi." - Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Brescia, 5th of February, 2016).
- **Biasato I.**, Biasibetti E., Spuria L., Cavallarin L., Gai F., Schiavone A., Capucchio M.T.: "Broiler chickens fed diets with *Tenebrio molitor* insect inclusion: histological and morphometric investigations." - XXVIII Reunion de la SEAPV (Cordoba, 08-09-10th of June, 2016).
- Vercelli C., **Biasato I.**, Dell'Armeline Rocha P.R., Capucchio M.T., Sossella M., Grego E., Candini D.: "Desmoplastic epithelioid olfactory neuroblastoma in a *Distemper Virus* infected dog." - LXX Convention S.I.S.Vet (Palermo, 13-14-15-16th of June, 2016).
- Tarantola M., **Biasato I.**, Biasibetti E., Biagini D., Guarda F., Maniscalco B., Mioletti S., Ricci A., Salaroglio A., Spuria L., Vincenti L., Capucchio M.T.: "On-farm welfare assessment in beef cattle: an integrated approach." - LXX Convention S.I.S.Vet (Palermo, 13-14-15-16th of June, 2016).
- Vergnano D., Biasato I., Capucchio M.T., Bruni N., Cocca T.: "Efficacy of dietary supplementation in cats with advanced chronic kidney disease." - LXX Convention S.I.S.Vet (Palermo, 13-14-15-16th of June, 2016).
- **Biasato I.**, Vergnano D., Capucchio M.T., Biasibetti E., Bruni N., Cocca T.: "Chronic kidney disease in dogs: tolerability and efficacy of a nutritional supplement." - LXX Convention S.I.S.Vet (Palermo, 13-14-15-16th of June, 2016).

- Capucchio M.T., Biasibetti E., **Biasato I.**, Barbero P., Bruni I., Spuria L., Mioletti S., Foglietta F., Querio G., Durando G., Canaparo R., Serpe L.: "Anticancer sonodynamic treatment with porphyrin compounds: insights on in vivo efficacy in a syngeneic rat model of cancer." - 34th Meeting of the European Society of Veterinary Pathology and 27th Meeting of the European College of Veterinary Pathologists (Bologna, 07-08-09-10th of September, 2016).
- Biasibetti E., Spuria L., Bisanzio D., **Biasato I.**, Bianco P., Caruso C., Di Blasio A., Lamberti M., Masoero L., Dondo A., Capucchio M.T.: "Epidemiological studies on biological agents in macroscopically healthy small ruminants mammary glands." - 34th Meeting of the European Society of Veterinary Pathology and 27th Meeting of the European College of Veterinary Pathologists (Bologna, 07-08-09-10th of September, 2016).
- **Biasato I.**, Biasibetti E., Spuria L., Schiavone A., Gasco L., Dall'Aglio C., Capucchio M.T.: "Histological, morphometric and histochemical findings in broiler chickens fed diets with insect meal inclusion." - 34th Meeting of the European Society of Veterinary Pathology and 27th Meeting of the European College of Veterinary Pathologists (Bologna, 07-08-09-10th of September, 2016).
- Spuria L., Sossella M., Mignacca S.A., Amato B., Grego E., Camperio C., Armas F., Biasibetti E., **Biasato I.**, Barbero P., Dore S., Cannas A., Di Marco Lo Presti V., Capucchio M.T., Marianelli C.: "Evaluation of the interleukin (IL)-1B and IL-8 gene expression in healthy sheep udders infused with live *Lactococcus lactis*: preliminary data." - 34th Meeting of the European Society of Veterinary Pathology and 27th Meeting of the European College of Veterinary Pathologists (Bologna, 07-08-09-10th of September, 2016).
- Spuria L., Di Blasio A., Caruso C., Bisanzio D., Biasibetti E., **Biasato I.**, Lamberti M., Bianco P., Masoero L., Dondo A., Capucchio M.T.: "MASTITI DEI PICCOLI RUMINANTI IN PIEMONTE: RILIEVI MICROBIOLOGICI ED ISTOPATOLOGICI." - XII National Congress S.I.P.A.O.C. (Cuneo, 13-14-15-16th of September, 2016).
- De Meneghi D., Spuria L., Biasibetti E., Robino P., Bisanzio D., Caruso C., Di Blasio A., Nebbia P., **Biasato I.**, Bianco P., Lamberti M., Masoero L., Dondo A., Capucchio M.T.: "Studies on potential agents of mastitis in udders from small ruminants regularly slaughtered in Piemonte Region, North-Western Italy (part I): microbiological and pathological findings." - Annual Scientific Conference and Annual General Meeting of the European College of Veterinary Public Health (Uppsala, 26-27-28th of September, 2016).

- Nebbia P., Robino P., De Meneghi D., Spuria L., Biasibetti E., Bisanzio D., Caruso C., Di Blasio A., **Biasato I.**, Bianco P., Lamberti M., Masoero L., Dondo A., Capucchio M.T.: "Studies on potential agents of mastitis in udders from small ruminants regularly slaughtered in Piemonte Region, North-Western Italy (part II): evaluation of antimicrobial resistance." - Annual Scientific Conference and Annual General Meeting of the European College of Veterinary Public Health (Uppsala, 26-27-28th of September, 2016).
- **Biasato I.**, Biasibetti E., Cenacchi G., Chiappino L., Sereno A., Arnulfo D., Evangelista R., Guarda F., Capucchio M.T.: "ANITSCHKOW CELLS IN VEAL CALVES AND BEEF CATTLE CORONARY ARTERIOSCLEROSIS: POTENTIAL ROLE IN THE DEVELOPMENT OF VASCULAR DEGENERATION." - LXXI Convention S.I.S.Vet (Napoli, 28-29-30th of June, 2017 - 01st of July, 2017).
- **Biasato I.**, Biasibetti E., Sereno A., Gai F., Gasco L., Schiavone A., Capucchio M.T.: "Intestinal mucin dynamics in different chicken strains fed diets with *Tenebrio molitor* inclusion." - 21st European Symposium of Poultry Nutrition (Salou-Vila-Seca, Terragona, 08-09-10-11th of May, 2017).
- Schiavone A., Dabbou S., De Marco M., Cullere M., **Biasato I.**, Biasibetti E., Capucchio M.T., Meneguz M., Gai F., Dalle Zotte A., Gasco L.: "Inclusion of black soldier fly (*Hermetia illucens* L.) larvae fat in finisher broiler chickens diets." - 21st European Symposium of Poultry Nutrition (Salou-Vila-Seca, Terragona, 08-09-10-11th of May, 2017).
- **Biasato I.**, Ferrocino I., Biasibetti E., Grego E., Schiavone A., Gasco L., Gai F., Cocolin L.S., Capucchio M.T.: "MODULATION OF INTESTINAL MICROBIOTA, MORPHOLOGY AND MUCIN COMPOSITION BY DIETARY INSECT MEAL INCLUSION IN FREE-RANGE CHICKENS." - 35th Meeting of the European Society of Veterinary Pathology and 28th Meeting of the European College of Veterinary Pathologists (Lyon, 30-31st of August, 2017 – 01-02nd of September, 2017).
- **Biasato I.**, Biasibetti E., Dellarole S., Miniscalco B., Mioletti S., Tarantola M., Biagini D., Capra P., Leporati M., Vincenti M., Guarda F., Capucchio M.T.: "INTRAMURAL CORONARY ARTERIOSCLEROSIS IN BEEF CATTLE REARED UNDER DIFFERENT HOUSING CONDITIONS." - 35th Meeting of the European Society of Veterinary Pathology and 28th Meeting of the European College of Veterinary Pathologists (Lyon, 30-31st of August, 2017 – 01-02nd of September, 2017).

- Tarantola M., **Biasato I.**, Biasibetti E., Biagini D., Capra P., Guarda F., Leporati M., Miniscalco B., Mioletti S., Salaroglio A., Vincenti M., Capucchio M.T.: "Welfare assessment in beef cattle: a multidisciplinary approach." - 7th International Conference on the Assessment of Animal Welfare at Farm and Group Level (Ede, 05-06-07-08th of September, 2017).

12.3 Congresses, Seminars and Awards

- LXIX Convention S.I.S.Vet – Department of Veterinary Medicine, University of Perugia - 15-16-17th of June, 2015. Oral Abstract Presenter.
- 34th Meeting of the European Society of Veterinary Pathology and 27th Meeting of the European College of Veterinary Pathologists – University of Bologna, Complesso Belmeloro - 07-08-09-10th of September, 2016. Poster Presenter.
- XII National Congress S.I.P.A.O.C. – Meeting Center of Cuneo Province - 13-14-15-16th of September, 2016. Participant.
- 21st European Symposium of Poultry Nutrition - Convention Centre PortAventura - Salou-Vila-Seca, Terragona, 08-09-10-11th of May, 2017). Poster Presenter.
- Seminario: "Hermetia illucens in zootecnia." – Department of Veterinary Sciences, University of Turin, Grugliasco (TO). 2nd of March, 2017 – Invited Speaker.
- ECM Course: "Utilizzo degli insetti in alimentazione animale: stato dell'arte e prospettive." – Department of Veterinary Sciences, University of Turin, Grugliasco (TO). 13th of October, 2017 – Invited Speaker.
- Best poster for the PhD Program within the D-Day 2017 hold on 19th of September, 2017 – Title: "Modulation of intestinal microbiota, morphology and mucin composition by dietary insect meal inclusion in free-range chickens."

12.4 Stays abroad

- 22nd of March, 2015 – 03rd of April, 2015: Training in histochemical and immunohistochemical techniques under the supervision of Prof. Cecilia Dell'Aglio – Veterinary Anatomy Section – University of Perugia.
- 15th of January, 2017 – 22nd of January, 2017: Training in histochemical and immunohistochemical techniques under the supervision of Prof. Cecilia Dell'Aglio - Veterinary Anatomy Section - University of Perugia.
- 3rd of March, 2017 – 3rd of July, 2017: Training in neuropathology and immunohistochemistry in animal neurological diseases under the supervision of Prof Martí Pumarola i Battle - Department of Animal Medicine and Surgery of the Universitat Autònoma de Barcelona (Spain).

ORIGINAL ARTICLE

Effects of dietary *Tenebrio molitor* meal inclusion in free-range chickens

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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. This 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, haematological 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, haematological or serum parameters. The morphometric and histological features were not significantly affected either, thus suggesting no influence on nutrient metabolism, 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

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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; Adeniji, 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; Adeniji, 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, 2013).

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 (440–690 g/kg) and fat (230–470 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 (Belforti *et al.*, 2015; Roncarati *et al.*, 2015).

Intestinal morphology is the main indicator of gut health and functioning (Kuzmuk *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 (Salamano *et al.*, 2010; De Marco *et al.*, 2013). 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 this study was to evaluate the effects of TM dietary inclusion on the productive performance, intestinal morphology, histological features, haematochemical 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 and 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, 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 (National Research Council (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- to 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

The diets were subsequently ground to pass through a 0.5-mm sieve and stored in airtight plastic containers for DM, ash, CP, crude fibre (AOAC, 2005) and EE (Folch et al., 1957) determination. The fatty acid composition of the control and TM diets was assessed using the method described by Alves et al. (2008). Fatty acid methyl esters were separated, identified and quantified on the basis of the chromatographic conditions reported by Renna et al. (2014). Heptadecanoic acid (C17:0) was used as the internal standard. The results were expressed in absolute values as g/kg DM.

Table 1 Ingredients and chemical composition of the experimental diets

	Control diet	TM diet
<i>Ingredients (g/kg as fed)</i>		
Corn meal	720.0	720.0
Soya bean meal	170.0	170.0
<i>Tenebrio molitor</i> meal	–	75.0
Gluten meal	75.0	–
Vitamin-mineral premix*	35.0	35.0
Metabolizable energy (MJ/kg)	12.18	12.22
<i>analysed composition</i>		
Chemical composition (g/kg DM)		
Dry matter	868	867
Crude protein	169	168
Crude fat	31	50
Crude fibre	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 vitamin A; 65.000 IU vitamin D3; 650 IU vitamin E; 80 mg vitamin K; 80 mg vitamin B1; 150 mg vitamin B2; 770 mg vitamin B3; 80 mg vitamin B6; 0.5 mg vitamin B12; 240 mg pantothenic acid; 4700 mg betaine; 1750 mg Iron (II) carbonate; 1835 mg Magnesium oxide; 1612 mg Zinc oxide; 178 mg Copper (II) oxide; 18.3 mg Potassium iodide; 6.6 mg Sodium selenite; 4100 mg DL-methionine; 5500 mg L-lysine; 120 g Calcium carbonate; 450 g Calcium phosphate; 11.5 g Sodium chloride. †SFA: saturated fatty acids. ‡MUFA: monounsaturated fatty acids. §PUFA: polyunsaturated fatty acids. ¶Other FAs (all <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.

To perform the AA determination, samples of the diets were prepared using a 22-h hydrolysis step in 6 HCl at 112 °C under a nitrogen atmosphere. Performic acid oxidation occurred prior to acid hydrolysis for methionine and cystine. The AA in the hydrolysate was determined by means of HPLC after post-column derivatization, according to the procedure described by Madrid et al. (2013). Tryptophan was not determined.

All the analyses were performed in duplicate.

Haematological and serum parameters

At the end of the experiment (day 97), blood samples were collected at slaughtering from 4 birds per pen: 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 leucocytes, including granular (heterophils, eosinophils and basophils) and non-granular (lymphocytes and monocytes) leucocytes, were counted on the slide, and the H/L ratio was calculated. The tubes without anticoagulant were left to clot in a standing position at room temperature for approximately two hours to obtain serum. The serum was separated by means of centrifugation at 700 *g* for 15 min 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 alanine-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 was recorded.

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, haemorrhage 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, Chicago, IL, USA; SPSS, 2008). The experimental unit was the pen. The influence of diet on the performance parameters, haematological and serum biochemical traits and intestinal morphometric measurements were analysed using Student's *t*-tests for independent samples. Morphometric data were also analysed 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,

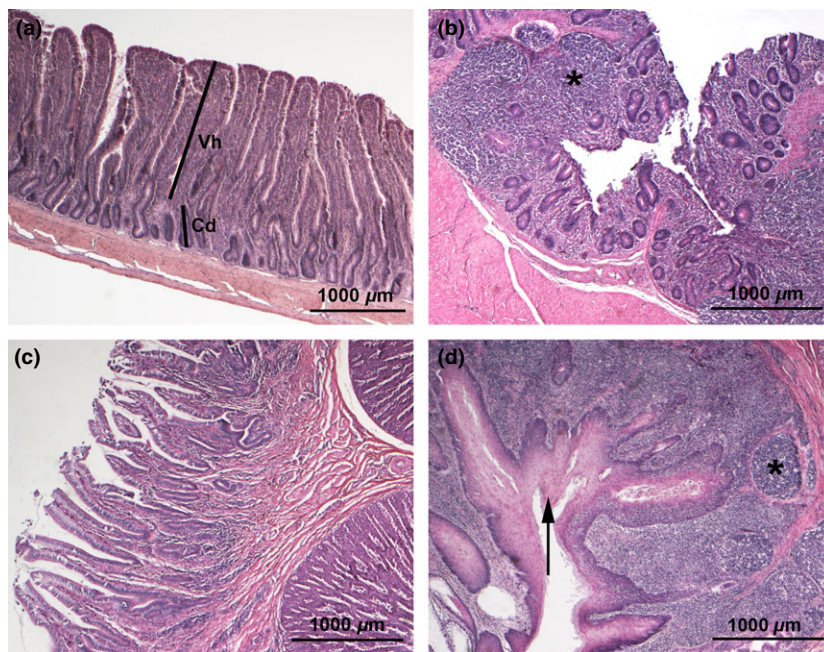


Fig. 1 Histological and morphometric evaluation of the gastrointestinal tract of the free-range chickens. (a) TM group. Morphometric measurements of the villus height (Vh) and the crypt depth (Cd) in the jejenum segment. 2.5× H&E. (b) TM group. Severe and diffuse mucosal lymphoplasmacytic and macrophagic flogosis with activation of the lymphoid tissue (*) in the caecum. 5× H&E. (c) C group. A normal glandular stomach. 5× H&E. (d) C group. Severe and diffuse mucosal lymphoplasmacytic flogosis with epithelial squamous metaplasia (arrow) and activation of the lymphoid tissue (*) in the glandular stomach. 5× H&E.

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-medullary ratio
Bursa of Fabricius	Follicular depletion	Number of apoptosis
	Intrafollicular cysts	Cell types
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

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- to 97-d 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 and 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 and b). Finally, the liver showed different degrees of lymphoid tissue activation

Table 3 Growth and slaughtering performance of the free-range chickens (mean $\pm$ SD)

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

Table 4 Haematological and serum biochemical traits of the free-range chickens (mean $\pm$ SD)

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

*H/L: heterophils/lymphocytes.

†AST: aspartate aminotransferase.

‡ALT: alanina-aminotransferase.

(Fig. 2c and 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 soya bean 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% soya bean meal in laying hens diets (Wang *et al.*, 2015).

Footpad dermatitis 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

Table 5 Intestinal morphometric measurements of the free-range chickens

	Control diet	TM diet	SEM	p
Duodenum				
1. Villus height (μ m)	2.29 ^a	2.49 ^a	0.06	0.126
2. Crypt depth (μ m)	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 (μ m)	1.94 ^b	1.93 ^b	0.08	0.945
2. Crypt depth (μ m)	0.17	0.17 ^e	0.01	0.813
3. Villus height/crypt depth ratio	11.29 ^g	11.14	0.44	0.875
Ileum				
1. Villus height (μ m)	1.60 ^c	1.72 ^b	0.07	0.405
2. Crypt depth (μ m)	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.

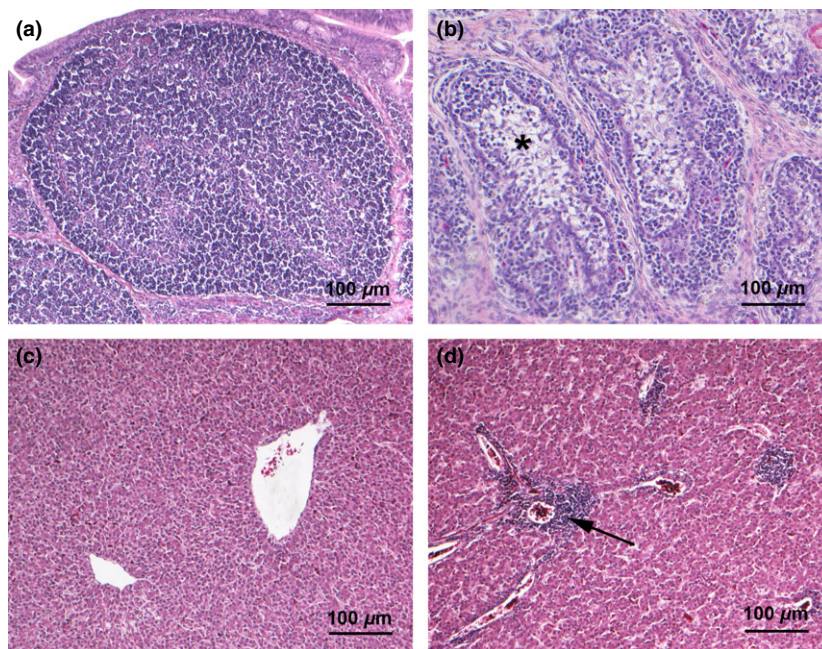


Fig. 2 Histological examination of the organs of the free-range chickens. (a) TM group. A normal follicle in the bursa of Fabricius. 10× H&E. (b) TM group. Severe and diffuse follicular depletion (*) in the bursa of Fabricius. 20× H&E. (c) C group. A normal liver. 10× H&E. (d) C group. Mild and multifocal perivascular activation of the lymphoid tissue (arrow) in the liver. 10× H&E.

assessment of broiler welfare in processing plants (Michel 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 bacteria that may spread haematogenously and impair product quality. Birds with severe lesions may also show reduced weight gains, due to pain-induced decreases in feed intake (Martland, 1985). FDP can be caused by several factors, the most important being the condition of the litter in the broiler house (Bessei, 2006). Litter quality can be affected by diet composition, as well as by the number and design of the drinkers in the pen (Lynn and Elson, 1990; Jones et al., 2005). The score obtained in the present study was zero for all of the birds, thus showing that they were in a good welfare condition.

Intestinal morphology is the main indicator of gut health and functioning (Kuzmuk et al., 2005). In the present study, mealworm inclusion did not affect the morphology of the small intestine, thus suggesting no influence on nutrient metabolism or performance. The greater development of the duodenum, in relation to the other intestinal segments, is in agreement with the results of Uni et al. (1999), Kondo (2003) and Murakami et al. (2007). In fact, the duodenum is the intestinal tract with the fastest cell renewal and is also the first segment of the small intestine to receive physical, chemical and hormonal stimuli provoked by

the presence of the diet in the lumen (Macari, 1998).

In the present study, mealworm inclusion did not induce histological changes, thus suggesting no negative influence on animal health. Glandular stomach alterations were considered paraphysiological because, in the authors' opinion, they were probably related to the free-range farming system. Outdoor access in fact entails less control of animal feeding, with the possible ingestion of foreign bodies. However, the activation of the lymphoid tissue, in both dietary treatments, represents the most interesting result. It has been reported that keeping birds in free-range systems may favour the occurrence of gastrointestinal parasitic diseases, with a high prevalence of coccidiosis and helminthiasis (Magwisha et al., 2002; Tomza-Marciniak et al., 2014). Although all the birds were vaccinated against coccidiosis, the 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.

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

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ABSTRACT

The aim of the present study was to evaluate the animal performance, haematochemical parameters, intestinal morphology and histological features of broiler chickens fed diets including *Tenebrio molitor* (TM) larvae meal. A total of 160 female broiler chicks (Ross 708) at one-day of age were randomly allotted to four dietary treatments: a control (C) group and three TM groups, in which TM meal was included at 50 (TM50), 100 (TM100) and 150 (TM150) 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 TM150) and quadratic response (40 days, $P < 0.05$, maximum with TM50) to dietary TM meal inclusion. The average daily gain (ADG) showed a linear increase (1–12 days, $P < 0.05$, maximum with TM150) in response to TM meal utilization. A linear effect (1–12 and 12–25 days, $P < 0.01$ and $P < 0.05$, maximum with TM150 and TM50) was observed for the daily feed intake (DFI). The feed conversion ratio (FCR) showed a linear response to TM utilization in the period 12–25 days ($P < 0.01$, maximum with TM150). A quadratic effect ($P < 0.05$, maximum with TM50) was observed for the carcass weight. The abdominal fat weight and percentage showed a linear response to dietary TM meal inclusion ($P < 0.05$ and $P < 0.01$, maximum with TM150 and TM100). A quadratic increase ($P < 0.05$, maximum with TM100) was observed for the erythrocytes, while the albumin and GGT showed a linear and quadratic decrease ($P < 0.05$, minimum with TM100) in relation to TM utilization. Gut morphology and histopathological findings were not significantly influenced ($P > 0.05$) by dietary TM meal inclusion. The present study suggests that increasing levels of dietary TM meal inclusion

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in female broiler chickens diets may improve body weight and feed intake, but can partially worsen feed efficiency. However, positive effects on carcass traits and haematochemical parameters related to TM meal utilization are observed, along with no negative influence on gut morphology and histological findings.

1. 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* have recently been used in poultry (Biasato et al., 2016; Bovera et al., 2016; Schiavone et al., 2017a) and fish (Belforti et al., 2015; Gasco et al., 2016; Renna et al., 2017) feeding. Yellow mealworms are already industrially produced as feed for pets and zoo animals, such as birds, reptiles, small mammals, amphibians and fish, and TM larvae are easily bred on dried waste materials, being

Table 1

Ingredients (g/kg as fed), apparent metabolizable energy (MJ/kg DM) and nutrient composition (%) of the experimental diets.

Ingredients	First Period (days 1–12)				Second period (days 12–25)				Third period (day 25 to slaughter)			
	Control	TM50	TM100	TM150	Control	TM50	TM100	TM150	Control	TM50	TM100	TM150
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 ^a	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 ^b (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 (%) ^b												
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

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

^a 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.

^b Calculated according to INRA, 2004 and De Marco et al. (2015).

able to recycle them into high-quality feed with less energy cost, land area utilization and footprints (Makkar et al., 2014). The influence of TM-based diets on growth performance (Bovera et al., 2015; Bovera et al., 2016; Biasato et al., 2016) haematochemical profile (Bovera et al., 2015; Biasato et al., 2016) and carcass traits (Ballitoc and Sun, 2013; Bovera et al., 2016; Biasato et al., 2016), has recently been investigated. Gut morphology, which has been reported to be widely affected by dietary modifications in broilers (Laudadio et al., 2012; Gopinger et al., 2014; Qaisrani et al., 2014), has also been evaluated in free-range chickens fed diets with dietary TM larvae meal inclusion (Biasato et al., 2016).

Despite insect meals being considered suitable ingredients for poultry feeding (Veldkamp et al., 2012; van Huis, 2013; Makkar et al., 2014), the implications of their utilization on poultry health and gut development are still very limited. The aim of the present study was to evaluate the growth performance, haematochemical parameters, carcass traits, intestinal morphology and histological features of female broiler chickens fed diets including TM meal.

2. Materials and methods

2.1. Birds and husbandry

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

A total of 160 female broiler chicks (Ross 708) at one-day of age were randomly allotted to 4 dietary treatments, each consisting of 5 pens as replicates with 8 chicks per pen. Each pen was 1.20 m wide × 1.20 m long and was equipped with a feeder occupying a surface of almost 1800 cm², three nipple drinkers and rice hulls as litter. During the first three weeks, the animals were heated by infrared lamps to maintain the suitable temperature according to standard breeding practices (Aviagen, 2014). Lighting schedule was 23 h light: 1 h darkness until day 3 and 18 h light: 6 h darkness until slaughter age. At hatching, all chicks received vaccination against Newcastle disease, Gumboro disease, infectious bronchitis and coccidiosis. Vaccine recalls were performed on day 9 for infectious bronchitis and on day 18 for Gumboro and Newcastle diseases.

2.2. Diets

A basal diet based on corn meal, corn gluten meal and soybean meal was formulated and served as control (C) group, while 50, 100 and 150 g/kg full-fat TM larvae meal (Gaobeidian Shannong Biology CO., LTD, Gaobeidian, Hebei province, China) inclusion as a partial replacement of soybean meal, corn gluten meal and soybean oil constituted the three experimental treatment groups (TM50, TM100 and TM150) (Table 1). TM meal nutritive composition and energy content were the following: dry matter, 939.0 (g/kg as fed); organic matter, 912.0 (g/kg as fed); crude protein (CP), 519.0 (g/kg as fed); ether extract (EE), 236.0 (g/kg as fed); 117.0 (g/kg as fed); neutral detergent fibre (NDF); 79.5 (g/kg as fed); acid detergent fibre (ADF); DL-methionine, 10.1 (g/kg as fed); L-lysine, 35.9 (g/kg as fed); gross energy, 24.4; apparent metabolizable energy (AMEn), 16.02 (MJ/kg DM). Three different diets were used per each dietary treatments during the three phases of growth: a starter diet (days 1–12), a grower diet (days 12–25) and a finisher diet (day 25–40). For each phase, the experimental diets were isonitrogenous and isoenergetic and were formulated using the AMEn values for TM measured in vivo for broiler chickens (De Marco et al., 2015). Diets met or exceeded NRC (1994) requirements and were adjusted according to Aviagen (2014) broiler nutrition specifications. Feed and water were provided *ad libitum*.

2.3. Chemical analysis

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

2.4. Growth performance

Health status and mortality were daily monitored during the whole experimental period. Live weight (LW) of the animals was recorded at an individual level at the beginning of the trial, at day 12, 25 and 40. Average daily gain (ADG) and average daily feed intake (DFI) were recorded at an individual and a pen level, respectively, at the end of each growth period. Feed conversion ratio (FCR) was determined for each growth period and for the overall experimental period. All measurements were made on the pen basis using a high precision electronic scale (Sartorius – Signum®).

2.5. Slaughtering procedure and recordings

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

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

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

2.6. 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 min 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 alanine-aminotransferase (ALT), aspartate-aminotransferase (AST), gamma glutamyl transferase (GGT), triglycerides, cholesterol, glucose, phosphorus, magnesium, iron, uric acid and creatinine serum concentrations were measured by means of enzymatic methods in a clinical chemistry analyzer (Screen Master Touch, Hospitex diagnostics Srl., Firenze, Italy).

2.7. Histomorphological investigations

The slaughtered animals were submitted to anatomopathological investigations. Intestinal segment samples (approximately 5 cm in length) of duodenum, jejunum and ileum were excised from each bird and flushed with 0.9% saline to remove all the content. The collected segments of intestine were the loop of the duodenum, the tract before Meckel's diverticulum (jejunum) and the tract before the ileocolic junction (ileum). Gut segments were fixed in Carnoy's solutions for morphometric analysis. Tissues were routinely embedded in paraffin wax blocks, sectioned at 5 μm thickness, mounted on glass slides and stained with Haematoxylin & Eosin (HE). The evaluated morphometric indices were Vh (from the tip of the villus to the crypt), Cd (from the base of the villus to the sub-mucosa) and the Vh/Cd ratio (Laudadio et al., 2012). Morphometric analyses were performed on 10 well-oriented and intact villi and 10 crypts chosen from duodenum, jejunum and ileum (Qaisrani et al., 2014). Samples of liver, spleen, thymus, bursa of Fabricius, kidney and heart were also collected from each animal and fixed in 10% buffered formalin solution for histopathological examination. Tissues were processed in the same way as gut and the following histopathological alterations were evaluated: white pulp hyperplasia and depletion in spleen, cortical depletion in thymus, follicular depletion and intrafollicular cysts in bursa of Fabricius and lymphoid tissue activation in liver (Biasato et al., 2016). Heart and kidney were assessed for inflammatory and degenerative diseases. The observed histopathological findings were evaluated using a semiquantitative scoring system as previously assessed by Biasato et al. (2016): absent/minimal (score = 0), mild (score = 1) and severe (score = 2).

2.8. Statistical analysis

IBM SPSS Statistics V20.0.0 software was used to perform statistical analysis. Shapiro-Wilk's test established normality or non-normality of distribution. The experimental unit was the pen for growth performance, haematochemical parameters and carcass traits and bird for histomorphological findings. Data collected for growth performance, blood parameters and carcass traits were tested by one-way ANOVA, evaluating the effect of dietary TM inclusion by polynomial contrasts. χ^2 test was performed to evaluate the association between the mortality rate and the dietary treatments. Intestinal morphometric indices were analyzed by fitting a general linear model (GLM). The GLM allowed the morphometric indices (Vh, Cd and Vh/Cd, separately) to depend on three fixed factors (diet, intestinal segment and interaction between diet and intestinal segment). The interactions between the levels of the fixed factors were evaluated by pairwise comparisons. Statistical analysis was performed by procedure “General Linear Models > Univariate”. Histopathological and FPD scores were analyzed by Kruskal-Wallis test (post-hoc test: Dunn's Multiple Comparison test). P values < 0.05 were considered statistically significant. The results were expressed as mean and pooled standard error of the mean (SEM).

3. Results

3.1. Growth performance

No clinical signs were observed and the birds remained healthy during the whole experimental period. The mortality rates of C

(2.5%), TM50 (2.5%), TM100 (0%) and TM150 (2.5%) groups were not significantly different among treatments ($P > 0.05$). Growth performance of the broiler chickens are summarized in Table 2. At 12 days of age, the LW increased linearly with increasing TM meal levels ($P < 0.05$) and the linear response increased to a maximum corresponding to the inclusion of 150 g/kg of TM meal. At 25 days of age, no significant effects related to TM meal utilization were observed. At 40 days of age, the LW showed quadratic response to increasing TM meal levels ($P < 0.05$), with a maximum corresponding to the inclusion of 50 g/kg of TM meal. In the period from 1 to 12 days of age, the ADG increased linearly with increasing TM meal levels ($P < 0.05$) and the linear response increased to a maximum corresponding to the inclusion of 150 g/kg of TM meal. On the contrary, the ADG showed no differences ($P > 0.05$) in the periods from 12 to 25 and 25–40 days of age. In the period from 1 to 12 days of age, the response of DFI to the effect of TM meal inclusion was statistically significant ($P < 0.01$). In particular, the linear response increased to a maximum corresponding to the inclusion of 150 g/kg of TM meal. In the period from 12 to 25 days of age, the DFI increased linearly with increasing TM meal levels ($P < 0.05$) and the linear response increased to a maximum corresponding to the inclusion of 50 g/kg of TM meal. On the contrary, the DFI showed no differences ($P > 0.05$) in the period from 25 to 40 days of age. In the periods from 1 to 12, 25–40 and 1–40 days of age, the FCR was similar among the dietary treatments ($P > 0.05$). Differently, the FCR showed linear response to increasing TM meal levels in the period from 12 to 25 ($P < 0.01$), with a maximum corresponding to the inclusion of 150 g/kg of TM meal.

3.2. Slaughtering performance and footpad dermatitis (FPD) score

Table 3 summarizes the slaughtering performance of the broiler chickens. The carcass weight increased quadratically with increasing TM meal levels ($P < 0.05$) and the quadratic response increased to a maximum corresponding to the inclusion of 50 g/kg of TM meal. The abdominal fat weight showed linear response to increasing TM meal levels ($P < 0.05$), with a maximum corresponding to the inclusion of 150 g/kg of TM meal. Similarly, the abdominal fat percentage increased linearly with increasing TM meal levels ($P < 0.01$) and the linear response increased to a maximum corresponding to the inclusion of 100 g/kg of TM meal. On the contrary, no significant effects related to TM meal utilization were observed for the other carcass traits ($P > 0.05$). FPD scores (C: 0.40; TM50: 0.20; TM100: 0.20; TM150: 0.00) were also not influenced by dietary TM meal inclusion ($P > 0.05$).

3.3. Haematological and serum parameters

Haematological and serum biochemical traits of the broiler chickens are summarized in Table 4. The erythrocytes increased quadratically with increasing TM meal levels ($P < 0.05$) and the quadratic response increased to a maximum corresponding to the inclusion of 100 g/kg of TM meal. The albumin showed linear response to increasing TM meal levels ($P < 0.05$), with a minimum corresponding to the inclusion of 100 g/kg of TM meal. Similarly, the GGT decreased quadratically with increasing TM meal levels ($P < 0.05$) and the quadratic response decreased to a minimum corresponding to the inclusion of 100 g/kg of TM meal. On the contrary, no significant effects related to TM meal utilization were observed for the other blood and serum parameters ($P > 0.05$).

3.4. Histomorphological investigations

The effects of the diet, intestinal segment and interaction between diet and intestinal segment on gut morphometric indices of the broiler chickens are shown in Tables 5 and 6. There was no influence of diet or interaction between diet and intestinal segment

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

Variable ^b	Age	Dietary treatments ^a				SEM	P [*]	
		C	TM50	TM100	TM150		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

^a Each mean represents 5 replicates with 8 chicks/replicate ($n = 40/\text{treatment}$). Four dietary treatments: C = control; TM50 = 50 g/kg inclusion level of *Tenebrio molitor*; TM100 = 100 g/kg inclusion level of *Tenebrio molitor*; TM150 = 150 g/kg inclusion level of *Tenebrio molitor*.

^b LW, live weight; DFI, daily feed intake; ADG, average daily gain; FCR, feed conversion ratio; n, number of pens.

^{*} Statistical significance: $P < 0.05$.

Table 3Effect of the dietary TM larvae meal inclusion on the carcass traits of the female broiler chickens.^a

Variable	Dietary treatments ^b				SEM	P ^c	
	C	TM50	TM100	TM150		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

^a Each mean represents 5 pens with 2 chicks/pen (n = 5/treatment).^b Four dietary treatments: C = control; TM50 = 50 g/kg inclusion level of *Tenebrio molitor*; TM100 = 100 g/kg inclusion level of *Tenebrio molitor*; TM150 = 150 g/kg inclusion level of *Tenebrio molitor*.^c Statistical significance: P < 0.05.**Table 4**Effect of the dietary TM larvae meal inclusion on the haematological and serum parameters of the female broiler chickens.^a

Variable ^c	Dietary treatments ^b				SEM	P ^c	
	C	TM50	TM100	TM150		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 (U/l)	26.86	22.13	21.61	25.56	1.05	0.629	0.046
AST (U/l)	189.48	227.18	217.11	211.06	8.62	0.494	0.229
ALT (U/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

^a Each mean represents 5 pens with 2 chicks/pen (n = 5/treatment).^b Four dietary treatments: C = control; TM50 = 50 g/kg inclusion level of *Tenebrio molitor*; TM100 = 100 g/kg inclusion level of *Tenebrio molitor*; TM150 = 150 g/kg inclusion level of *Tenebrio molitor*.^c H/L, heterophiles to lymphocytes ratio; GGT, gamma glutamyl transferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; n, number of birds.^c Statistical significance: P < 0.05.

(P > 0.05) on the gut morphometric indices. On the contrary, Vh, Cd and Vh/Cd depended on intestinal segment (P < 0.001, P = 0.001 and P < 0.001, respectively) (Table 5). In particular, the duodenum showed higher Vh (P < 0.001) than the jejunum and ileum. Furthermore, higher Cd (P < 0.001) was found in the duodenum and jejunum than the ileum. Similarly, the duodenum and jejunum showed higher Vh/Cd (P < 0.001) than the ileum (Table 6).

Histopathological alterations were observed in all the dietary treatments and developed in spleen, thymus, bursa of Fabricius and liver, while heart and kidney showed no significant findings. Spleen (C = 1.50 ± 0.22; TM50 = 1.20 ± 0.25; TM100 = 1.10 ± 0.31; TM150 = 1.10 ± 0.28), thymus (C = 0.10 ± 0.10; TM50 = 0.30 ± 0.15; TM100 and TM150 = 0.10 ± 0.10), bursa of Fabricius (C = 1.90 ± 0.10; TM50 = 1.50 ± 0.27; TM100 = 1.80 ± 0.20; TM150 = 1.70 ± 0.21) and liver (C = 0.90 ± 0.23; TM50 = 1.10 ± 0.23; TM100 = 0.90 ± 0.23; TM150 = 0.30 ± 0.15) scores were not influenced by dietary TM meal inclusion. Spleen showed moderate (C = 30% of the broilers; TM50 = 40%; TM100 = 10%; TM150 = 30%) to severe (C = 60%; TM50 = 40%; TM100 = 50%; TM150 = 40%) white pulp depletion or hyperplasia. The 10% (C), 20% (TM50), 40% (TM100) and 30% (TM150) of the animals had normal spleen (Fig. 1A-B). In thymus,

Table 5

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

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

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

^a Four dietary treatments: C = control; TM50 = 50 g/kg inclusion level of *Tenebrio molitor*; TM100 = 100 g/kg inclusion level of *Tenebrio molitor*; TM150 = 150 g/kg inclusion level of *Tenebrio molitor*.^b Three intestinal segments: duodenum, jejunum and ileum.^c Degrees of freedom.^{*} Statistical significance: P < 0.05.**Table 6**

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

Index	Fixed effect	Effect levels	Least square mean ¹	SEM
Vh (mm)	Diet ²	C	1.73	0.06
		TM50	1.67	
		TM100	1.57	
		TM150	1.61	
	Intestinal segment ³	DU	2.08 ^a	0.05
		JE	1.65 ^b	
		IL	1.20 ^c	
Cd (mm)	Diet	C	0.20	0.01
		TM50	0.20	
		TM100	0.20	
		TM150	0.21	
	Intestinal segment	DU	0.21 ^a	0.00
		JE	0.21 ^a	
		IL	0.19 ^b	
Vh/Cd (mm/mm)	Diet	C	8.49	0.34
		TM50	8.51	
		TM100	8.00	
		TM150	8.00	
	Intestinal segment	DU	10.06 ^a	0.47
		JE	8.13 ^b	
		IL	6.56 ^c	

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

¹ Means with different superscript letters (a, b) within the same column per fixed effect (i.e. diet, intestinal segment) differ significantly (P < 0.05).² C = control; TM50 = 50 g/kg inclusion level of *Tenebrio molitor*; TM100 = 100 g/kg inclusion level of *Tenebrio molitor*; TM150 = 150 g/kg inclusion level of *Tenebrio molitor*.³ DU = duodenum; JE = jejunum; IL = ileum.

moderate cortical depletion was found in all the groups (C = 10% of the broilers; TM50 = 30%; TM100 and TM150 = 10%). A normal thymus was observed in 90% (C), 70% (TM50) and 90% (TM100 and TM150) of the animals. Bursa of Fabricius showed moderate (C and TM50 = 10% of the broilers; TM100 = 0%; TM150 = 10%) to severe (C = 80%; TM50 = 70%; TM100 = 90%; TM150 = 80%) follicular depletion. The 0% (C), 20% (TM50), 10% (TM100) and 10% (TM150) of the animals had normal bursa of Fabricius (Fig. 1C-D). In liver, moderate (C, TM50 and TM100 = 50% of the broilers; TM150 = 30%) to severe (C = 20%; TM50 = 30%; TM100 = 20%; TM150 = 0%) perivascular lymphoid tissue activation. A normal liver was observed in 30% (C), 20% (TM50), 30% (TM100) and 70% (TM150) of the animals.

4. Discussion

4.1. Growth performance

Growth performances of the broiler chickens of the present study were consistent with the reference values recorded in the

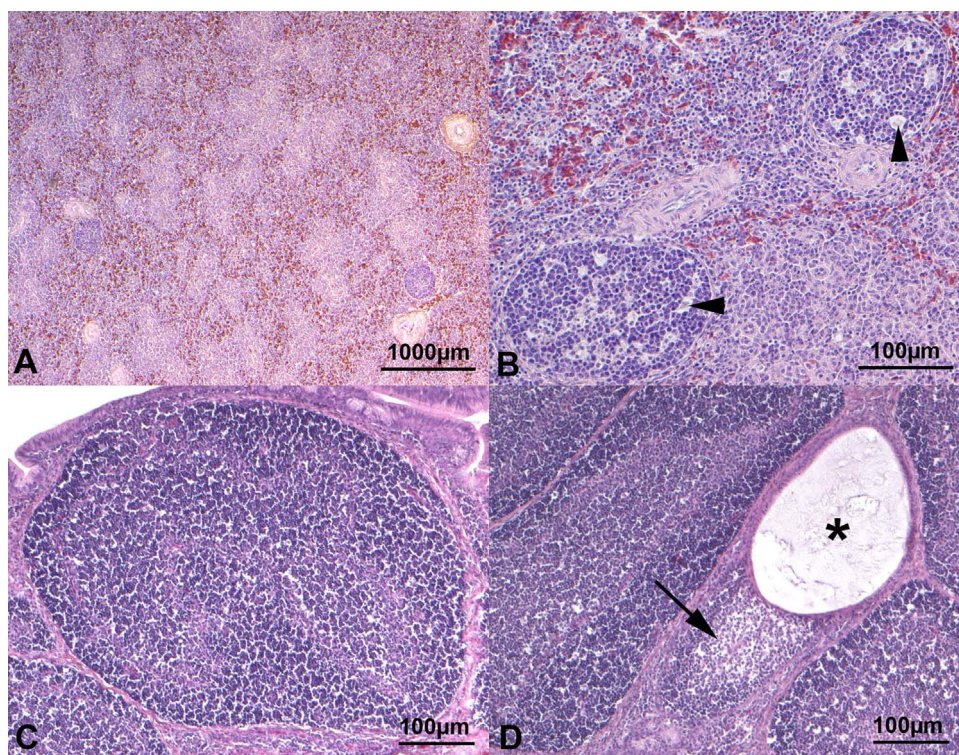


Fig. 1. Histological findings of the female broiler chickens. (A) TM50 group. A normal spleen. 5 × Haematoxylin & Eosin stain. (B) TM50 group. Spleen with severe and diffuse depletion of the white pulp. A high number of apoptotic cells (arrowheads) are observed. 20 × Haematoxylin & Eosin stain. (C) C group. A normal follicle in the bursa of Fabricius. 10 × Haematoxylin & Eosin stain. (D) C group. Bursa of Fabricius with mild and multifocal follicular depletion (arrow) associated with intrafollicular cyst (*). 10 × Haematoxylin & Eosin stain.

commercial farm in which the trial was conducted.

The body weight, weight gain and feed intake of the birds in the present trial improved with increasing levels of TM meal inclusion, but the feed efficiency resulted partially impaired. Little information on the influence of dietary TM meal inclusion in broiler chickens is currently available. Ramos-Elorduy et al. (2002) and Biasato et al. (2016) did not show any effects for the growth performance in fast-growing and intermediate-growing chickens, respectively, fed diets in which the TM inclusion level ranged from 50 to 100 g/kg. Differently, Ballitoc and Sun (2013) and Bovera et al. (2015) observed improved growth performance in fast-growing chickens fed diets with low (from 5 to 100 g/kg) or high (296 g/kg) TM inclusion levels, respectively. Similar findings were obtained by Loponte et al. (2017) in partridges fed diets in which the TM inclusion level ranged from 250 to 500 g/kg. Recently, Islam and Yang (2017) also found a positive effect of a mealworm-based probiotic on broiler growth performance. Some authors also explored the possibility to use other insect meals in poultry feeding. Adeniji (2007) and Hwangbo et al. (2009) studied the effects of housefly-maggots as feed supplement in the diet of broiler chickens: the first found no differences for growth performance with inclusion levels ranging from 55 to 220 g/kg, while the latter (inclusion rate: 50–200 g/kg) observed a linear increase in LW gain. Ijaiya and Eko (2009) evaluated the effects of replacing dietary fishmeal with silkworm meal (inclusion rate: 22–93 g/kg) on growth performance of broiler chickens, finding no differences related to insect meal utilization. Also Oyegoke et al. (2006) and Wang et al. (2005) observed no adverse effects on growth performance of broiler chickens fed diets with *Cirina forda* (inclusion rate: 20–40 g/kg) and *Gryllus testaceus* (inclusion rate: 50–150 k/kg), respectively. Cullere et al. (2016) recently studied the influence of the inclusion (from 100 to 150 g/kg) of *Hermetia illucens* meal in quail diet, finding no differences for growth performances. Schiavone et al. (2017b) also evaluated the effects of replacing soybean oil with *Hermetia illucens* meal (inclusion rate: 500–1000 g/kg) on growth performance of broiler chickens, finding no differences related to insect meal utilization. The wide variability of the results obtained in the previous studies may be related to the nutritive value of the insect meal used, which can be influenced by the species, the insect life stage (adult, larva or pupa) and the insect rearing substrate (Sánchez-Muros et al., 2014).

The improvement of feed intake observed in the birds fed TM diets of the present trial was considered suggestive of increased feed palatability in relation to the addition of yellow mealworms, since insects are naturally consumed by wild birds and free-range poultry (Zuidhof et al., 2003). In particular, the increased DFI observed in the starter period, which was accompanied by increased LW and ADG and unaffected FCR, was quite relevant. Indeed, starter period (from hatch to 10 days) is considered the most important in broiler production, since growth and development take place at an incredible rate during it. In this period, the chicks' weight quadruples, thus influencing the following growth rate (Aviagen, 2014). On the contrary, the increased DFI observed in the growing period was accompanied by unaffected LW and ADG and subsequently impaired FCR, thus representing a negative effect related to

TM meal utilization. De Marco et al. (2015) speculated that the chitin contained in the exoskeleton of the TM meal may negatively influence the apparent digestibility coefficient of the total tract of nutrients. Furthermore, Ravindran and Blair (1993) pointed out that the chitin of insects is difficult to digest by domestic poultry. As suggested by Rumpbold and Schülter (2013), the partial chitin removal through high pressure processing could improve the use of insects as feeding ingredient thanks to disruption of the link between some chitin-bound proteins. However, the limited number of birds included in the current trial could have influenced the data interpretation. The results obtained need to be confirmed on a larger number of animals.

4.2. 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. (2017a) 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 mealworm utilization may improve fat mass in broiler chickens (USDA, 2011).

The majority of FPD scores obtained in the present trial was zero and no differences were found in relation to TM meal utilization. This is a positive result, since a low prevalence and severity of FPD is highly desirable as far as health of birds and product quality are concerned (Meluzzi et al., 2008).

4.3. Haematological and serum parameters

All the blood parameters obtained in the present trial fell within the physiological ranges (Lumej, 2008), thus suggesting that TM meal utilization does not affect health status of the animals. In particular the H/L ratio, that is commonly used as indicator of stress in poultry (De Marco et al., 2013; Salamano et al., 2010), was not affected by dietary TM meal inclusion. As already observed by Bovera et al. (2015) and Biasato et al. (2016), the majority of the haematochemical and serum biochemical traits were not affected by yellow mealworm inclusion in the birds of the present study. Similar findings were obtained by Schiavone et al. (2017b) in broilers fed diets with *Hermetia illucens* fat. However, the erythrocytes increased and albumin and GGT decreased with increasing levels of TM meal utilization. Loponte et al. (2017) also observed lower albumin when TM and *Hermetia illucens* meals were included in the diets of partridges. Interestingly, this finding was accompanied by the increase of albumin/globulin ratio, which was also reported by Bovera et al. (2015) and ascribed to the properties of chitin contained in insect meal. High globulin concentrations and low albumin/globulin ratios generally indicate better disease resistance and immune response of birds (Griminger and Scanes, 1986). Another interesting finding is represented by the decrease of GGT serum levels in TM animals. Indeed, a high GGT concentration in birds is used as an indicator of liver disease and bile flow disorders (Ognik and Krauze, 2016). Therefore, GGT reduction can be considered a positive effect related to TM meal utilization.

4.4. Histomorphological investigations

Dietary TM meal inclusion did not influence the gut morphology of the birds of the present study, as already observed by Biasato et al. (2016). Morphometric measurements of Vh and Cd are generally used to assess intestinal development (Franco et al., 2006), since represent useful indicators of gut proliferative and absorptive compartments (Lenhardt and Mozes, 2003). The Vh/Cd ratio is also evaluated, because it gives an indication of the likely maturity and functional capacity of the enterocytes (Hampson, 1986). As previously reported (Uni et al., 1999; Iji et al., 2001; Biasato et al., 2016), the present study confirms that both duodenum and jejunum show a greater morphological development compared with the ileum. Indeed, the duodenum is the intestinal tract with the fastest cell renewal, and is also the first segment of the small intestine to receive physical, chemical and hormonal stimuli provoked by diet (Macari, 1998). Furthermore, the jejunum is an important site for nutrient digestion (Iji et al., 2001). Therefore, dietary TM inclusion preserves a proximodistal decreasing gradient of the morphometric indexes from the duodenum to the ileum, thus suggesting the maintenance of the physiological gut development.

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

5. Conclusion

In conclusion, the present study suggests that increasing levels of dietary TM meal inclusion in female broiler chickens diets may

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

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Yellow mealworm larvae (*Tenebrio molitor*) inclusion in diets for male broiler chickens: effects on growth performance, gut morphology, and histological findings

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ABSTRACT This study evaluated the effects of *Tenebrio molitor* (TM) larvae meal inclusion in diets for broilers. A total of 160 male 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 (TM5), 100 (TM10), and 150 (TM15) g/kg, respectively. The experimental diets were isonitrogenous and isoenergetic. Each group consisted of five pens as replicates (8 chicks/pen). After the evaluation of growth performance and haematochemical parameters, the animals were slaughtered at 53 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 and 25 days, $P < 0.001$ and $P < 0.05$, maximum with TM15 and TM10) and quadratic (53 days, $P < 0.05$, maximum with TM5) response to dietary TM meal inclusion. A

linear (1 to 12 and 12 to 25 days, $P < 0.001$, maximum with TM15) and quadratic (12 to 25 days, $P = 0.001$, maximum with TM15) effect was also observed for the daily feed intake (DFI). The feed conversion ratio (FCR) showed a linear response (25 to 53 and 1 to 53 days, $P = 0.001$ and $P < 0.05$, maximum with TM15). Haematological and serum biochemical traits, carcass traits and histopathological findings were not affected by dietary TM meal inclusion ($P > 0.05$). TM15 birds showed lower villus height ($P < 0.05$), higher crypt depth ($P < 0.05$), and lower villus height to crypt depth ratio ($P = 0.001$) compared with C and TM5. In conclusion, increasing levels of dietary TM meal inclusion in male broiler chickens may improve body weight and feed intake, but negatively affect feed efficiency and intestinal morphology, thus suggesting that low levels may be more suitable. However, no effect on haematochemical parameters, carcass traits, and histological findings were observed in relation to TM meal utilization.

Key words: broiler chickens, *Tenebrio molitor*, insect meal, growth performance, morphometry

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INTRODUCTION

The market for poultry products (egg and meat) is growing worldwide owing also to the absence of cultural or religious obstacles. Protein sources represent the primary production costs in poultry diets (FAO, 2013). Soybean meal is the most used vegetable protein source in diet formulations for broilers and lay-

ing hens, because of the high quality and quantity of protein and the adequate amino acid profile (Veldkamp et al., 2012). However, due to its ever-increasing price, the sustainability of this production chain is becoming critical, in particular in some developing countries. Fishmeal has been widely used in poultry nutrition in the last decades, due to its excellent nutritive properties related to its very high nutrient density and digestibility. The protein in fishmeal has high biological value, because it is rich in essential amino acids, particularly lysine and sulphur-containing amino acids. In some developing countries, fishmeal is the most

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important conventional animal protein source, but its production availability and cost is a major issue (FAO, 2013). Severe environmental concerns must also be considered in relation to the massive use of soybean and fishmeal. On one hand, the increased soy cultivation causes deforestation, high water consumption and utilization of chemicals. On the other hand, fishmeal quantitatively and qualitatively depends on catch, which is destined to decrease in the coming years due to the risk of marine resources depletion (Veldkamp et al., 2012; Sánchez-Muros et al., 2014).

Some studies have recently shown the feasibility of insect-derived feeds as a useful alternative to soybean and fishmeal in animal nutrition (van Huis, 2013; Makkar et al., 2014; Belforti et al., 2015; Henry et al., 2015; Gasco et al., 2016). Certain insect species are efficient feed converters because they do not use energy to maintain a high body temperature (Sánchez-Muros et al., 2014). Furthermore, they can be packed into land much more intensively, are highly digestible and can even be fed waste products which would otherwise be environmental problems (FAO, 2013; Makkar et al., 2014; Sánchez-Muros et al., 2014). Furthermore, insects (adult, larval and pupal forms) are naturally consumed by wild birds and free-range poultry (Zuidhof et al., 2003; FAO, 2013). In the European Union, insects and insect meals are currently only authorized for pet animals and farm fish. The partial or total replacement of soybean or fish meal by black soldier flies (*Hermetia illucens* L.) (Maurer et al., 2015; Cullere et al., 2016), houseflies (*Musca domestica* L.) (Hwganbo et al., 2009), mealworms (*Tenebrio molitor* L.) (Ballitoc and Sun, 2013; Bovera et al., 2015; Biasato et al., 2016; Bovera et al., 2016), and silkworms (*Bombyx mori* L.) (Khatun et al., 2003; Ijaiya and Eko, 2009) has recently been investigated in poultry feeding. Some authors evaluated the effects of TM meal utilization on growth performance (Ballitoc and Sun, 2013; Bovera et al., 2015; Bovera et al., 2016), haematochemical profile (Bovera et al., 2015), and carcass traits (Ballitoc and Sun, 2013; Bovera et al., 2016) of broilers, while others focused their attention also on the assessment of intestinal morphology and histological features in free-range chickens fed diets including TM meal (Biasato et al., 2016). Despite dietary modifications being reported to widely affect gut morphology in poultry (Laudadio et al., 2012; Qaisrani et al., 2014), no studies in relation to insect meal utilization are currently available in broilers. The aim of the present study was to evaluate growth performance, haematochemical parameters, carcass traits, intestinal morphology, and histological features of male broiler chickens fed diets including TM meal.

MATERIALS AND METHODS

Birds and Husbandry

The present trial was performed in collaboration with a local poultry corporation named “O.R.A. Agricola

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

A total of 160 male broiler chicks (Ross 708) at one-day of age were randomly allotted to 4 dietary treatments, each consisting of 5 pens as replicates with 8 chicks per pen. Each pen was 1.20 m wide × 1.20 m long and was equipped with a feeder occupying a surface of almost 1,800 cm², three nipple drinkers and rice hulls as litter. During the first 3 weeks, the animals were heated by infrared lamps to maintain the suitable temperature according to standard breeding practices (Aviagen, 2014). Lighting schedule was 23 h light:1 h darkness until day 3 and then 18 h light:6 hours darkness until slaughter age. At hatching, all chicks received vaccination against Newcastle disease, Gumboro disease, infectious bronchitis, and coccidiosis. Vaccine recalls were performed on day 9 for infectious bronchitis and on day 18 for Gumboro and Newcastle diseases.

Diets

A diet based on corn meal, corn gluten meal, and soybean meal was formulated and served as control (C), while 5, 10, and 15% full-fat TM larvae meal (Gaobeidian Shannong Biology CO., LTD, Gaobeidian, Hebei province, China) inclusion as a partial replacement of soybean meal, corn gluten meal and soybean oil constituted the 3 experimental treatment groups (TM5, TM10, and TM15) (Table 1). TM meal nutritive composition was previously analyzed as reported by De Marco et al. (2015) and summarized as follows: dry matter (DM), 948.0 (g/kg as fed); organic matter, 912.0 (g/kg as fed); crude protein (CP), 524.0 (g/kg as fed); ether extract (EE), 280.0 (g/kg as fed); neutral detergent fiber (NDF), 117.0 (g/kg as fed); acid detergent fibre (ADF), 79.5 (g/kg as fed); methionine, 5.9 (g/kg as fed); lysine, 14.7 (g/kg as fed); gross energy, 24.4 (MJ/kg DM); apparent metabolizable energy (AMEn), 16.02 (MJ/kg DM). The amount of chitin of the TM meal (46.2 g/kg as fed) was previously estimated by Bovera et al. (2016) as follows: chitin (%) = ash free ADF (%)—residual nitrogen in ADF × 6.25 (%) (Finke, 2007). For each dietary treatment, diets were divided into 3 phases: a starter diet (days 1 to 12), a grower diet (days 12 to 25), and a finisher diet (days 25 to 53). For each phase, the experimental diets were isonitrogenous and isoenergetic and were formulated using the AMEn values for TM calculated in vivo

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

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 larvae 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 ²	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Choline chloride	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 ³ (MJ/kg DM)	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

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

²Mineral-vitamin premix (Final B Prisma, IZA SRL, Forlì, Italy), 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.

³Calculated according to Sauvante et al. (2004) (ingredients AMEn, calcium, available phosphorus) and De Marco et al. (2015) (insect AMEn, digestible methionine, digestible lysine and digestible threonine).

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

for broiler chickens (De Marco et al., 2015). Diets met or exceeded (NRC 1994) requirements and were adjusted according to Aviagen (2014) broiler nutrition specifications. Feed and water were provided ad libitum.

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

Growth Performance

The trial lasted 53 days. Health status and mortality were daily monitored during the whole experimental period. Live weight (LW), daily feed intake (DFI), and feed conversion ratio (FCR) were determined for each diet phase and for the overall experimental period. All

measurements were made on pen basis using a high precision electronic scale (Sartorius—Signum).

Pre-slaughter Procedures

At day 53, all birds were individually weighed and 10 broilers/diet (2 birds/pen) were chosen on the basis of pen average LW and identified with a shank ring.

Haematological and Serum Parameters

At slaughter, blood samples of ten birds (two animals per pen) per feeding group 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). The alanine aminotransferase (**ALT**), aspartate aminotransferase (**AST**), gamma glutamyl transferase (**GGT**), triglycerides, cholesterol, glucose, phosphorus, magnesium, iron, uric acid, and creatinine serum concentrations were measured by means of enzymatic methods in a clinical chemistry analyzer (Screen Master Touch, Hospitex diagnostics Srl., Firenze, Italy).

Carcass Traits

At the end of the trial, ten selected chickens (two birds per pen) per feeding group were euthanized by electrical stunning and bleeding 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 liver, spleen, gizzard, and abdominal fat were immediately recorded. All slaughtered carcasses were stocked in a cooling chamber (0 to 4°C) for 24 h. Weights of carcass-for-grilling, breast and thighs were successively recorded. Carcass-for-grilling, breast, thighs, and organs weights were also expressed as percentage of LW.

Histomorphological Investigations

Ten birds per feeding group (two animals per pen) were submitted to anatomopathological investigations. Intestinal segment samples (approximately 5 cm in length) of duodenum, jejunum, and ileum were excised and flushed with 0.9% saline to remove all the content. The collected segments of intestine were the loop of the duodenum, the tract before Meckel's diverticulum (jejunum) and the tract before the ileocolic junction (ileum). Samples of liver, spleen, thymus, bursa of Fabricius, kidney, and heart were also collected. Gut segments were fixed in Carnoy's solution for morphometric analysis, while the other organ samples were fixed in 10% buffered formalin solution for histopathological examination. Tissues were routinely embedded in paraffin wax blocks, sectioned at $5\ \mu\text{m}$ thickness, mounted

on glass slides and stained with Haematoxylin & Eosin (**HE**). 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 villus to the submucosa), and the villus height to crypt depth (**Vh/Cd**) ratio (Laudadio et al., 2012). Morphometric analyses were performed on 10 well-oriented and intact villi and 10 crypts chosen from duodenum, jejunum, and ileum (Qaisrani et al., 2014). The following histopathological alterations were evaluated: white pulp hyperplasia and depletion in spleen, cortical depletion in thymus, follicular depletion and intrafollicular cysts in bursa of Fabricius, and lymphoid tissue activation in liver (Biasato et al., 2016). Heart and kidney were assessed for inflammatory and degenerative diseases. The observed histopathological findings were evaluated using a semi-quantitative scoring system as previously assessed by Biasato et al. (2016): absent/minimal (score = 0), mild (score = 1), and severe (score = 2).

Statistical Analysis

IBM SPSS Statistics V20.0.0 software was used to perform statistical analysis. Shapiro-Wilk's test established normality or non-normality of distribution. All data from each pen were considered the experimental unit. Data collected for growth performance, blood parameters, and carcass traits were tested by one-way ANOVA, evaluating the effect of dietary TM inclusion by polynomial contrasts. χ^2 test was performed to evaluate the association between the mortality rate and the dietary treatments. Intestinal morphometric indices were analyzed by fitting a general linear model (**GLM**). The GLM allowed the morphometric indices (Vh, Cd, and Vh/Cd, separately) to depend on three fixed factors (diet, intestinal segment, and interaction between diet and intestinal segment). The interactions between the levels of the fixed factors were evaluated by pairwise comparisons. Statistical analysis was performed by procedure “General Linear Models > Univariate”. Histopathological scores were analyzed by Kruskal-Wallis test (post hoc test: Dunn's Multiple Comparison test). *P* values < 0.05 were considered statistically significant. Results were expressed as mean and pooled standard error of the mean (**SEM**).

RESULTS

Growth Performance

Birds remained healthy during the whole experimental period. The mortality rates of C (5%), TM5 (5%), TM10 (2.5%), and TM15 (2.5%) groups were not affected by dietary treatment ($P > 0.05$). Growth performance of the broiler chickens are summarized in Table 2. At 12 and 25 days of age, the LW increased linearly with increasing TM meal levels ($P < 0.001$ and $P < 0.05$, respectively) and the linear response increased to a maximum corresponding to the inclusion of

Table 2. Effect of the dietary TM larvae meal inclusion on the growth performance of the male broiler chickens.¹

Variable ³	Age	Dietary treatments ²				SEM	P ⁴	
		C	TM5	TM10	TM15		Linear	Quadratic
LW (g)	12 d	287	308	350	358	10.12	<0.001	0.57
	25 d	1,105	1,129	1,272	1,253	43.80	0.018	0.67
	53 d	3,260	3,641	3,463	3,373	92.76	0.73	0.037
DFI (g)	1 to 12 d	23.8	30.0	30.5	35.6	0.75	<0.001	0.51
	12 to 25 d	81.4	111	104	113	2.55	<0.001	0.001
	25 to 53 d	184	220	209	225	11.65	0.06	0.43
FCR (g/g)	1 to 12 d	1.06	1.24	1.08	1.24	0.05	0.13	0.82
	12 to 25 d	1.33	1.78	1.47	1.73	0.10	0.07	0.38
	25 to 53 d	2.23	2.54	2.68	2.97	0.12	0.001	0.93
	1 to 53 d	1.92	2.26	2.18	2.46	0.11	0.010	0.80

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

²Four dietary treatments: C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*.

³LW, live weight; DFI, daily feed intake; FCR, feed conversion ratio; n, number of pens.

⁴Statistical significance: $P < 0.05$.

Table 3. Effect of the dietary TM larvae meal inclusion on the haematological and serum parameters of the male broiler chickens.¹

Variable ³	Dietary treatments ²				SEM	P ⁴	
	C	TM5	TM10	TM15		Linear	Quadratic
Erythrocyte (10^6 cell/ μ L)	2.52	2.36	2.39	2.38	0.66	0.56	0.61
Leukocyte (10^3 cell/ μ L)	8.12	9.06	8.34	8.03	0.45	0.82	0.53
H/L ratio	0.82	0.83	0.89	0.83	0.03	0.73	0.62
Albumin (g/dL)	1.12	1.35	0.99	1.06	0.06	0.25	0.43
Total protein (g/dL)	3.40	3.71	3.43	3.13	0.20	0.57	0.48
GGT (UI/L)	23.04	20.64	21.07	19.48	0.89	0.23	0.83
AST (UI/L)	336.17	337.37	292.75	246.86	18.01	0.06	0.51
ALT (UI/L)	9.56	11.35	10.44	11.41	0.75	0.52	0.80
Uric acid (mg/dL)	5.85	4.12	4.85	4.32	0.33	0.20	0.36
Creatinine (mg/dL)	0.39	0.38	0.38	0.37	0.01	0.77	0.87
Triglycerides (mg/dL)	65.83	52.70	58.94	59.91	2.83	0.66	0.24
Cholesterol (mg/dL)	97.93	122.75	98.70	91.31	4.60	0.24	0.06
Glucose (mg/dL)	227.90	231.50	228.00	229.70	2.99	0.95	0.89
Phosphorus (mg/dL)	5.02	5.49	4.50	5.34	0.34	0.59	0.34
Magnesium (mEq/L)	1.49	1.15	1.02	1.32	0.10	0.30	0.80
Iron (μ g/dL)	88.97	110.28	101.37	81.62	8.12	0.48	0.81

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

²Four dietary treatments: C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*.

³H/L, heterophiles to lymphocytes ratio; GGT, gamma glutamyl transferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; n, number of birds.

⁴Statistical significance: $P < 0.05$.

15 and 10% of TM meal, respectively. At 53 days of age, the LW showed quadratic response to increasing TM meal levels ($P < 0.05$), with a maximum corresponding to the inclusion of 5% of TM meal. In the period from 1 to 12 days of age, the response of DFI to the effect of TM meal inclusion was statistically significant ($P < 0.001$). In particular, the linear response increased to a maximum corresponding to the inclusion of 15% of TM meal. In the period from 12 to 25 days of age, the DFI increased linearly and quadratically with increasing TM meal levels ($P < 0.001$ and $P = 0.001$, respectively) and the linear and quadratic responses increased to a maximum corresponding to the inclusion of 15% of TM meal. On the contrary, the DFI showed no differences ($P > 0.05$) in the period from 25 to 53 days of age. In the periods from 1 to 12 and 12 to 25 days of age, the FCR was similar among the dietary treatments ($P > 0.05$). Differently, the FCR showed linear response

to increasing TM meal levels in the periods from 25 to 53 ($P = 0.001$) and 1 to 53 days of age ($P < 0.05$), with a maximum corresponding to the inclusion of 15% of TM meal.

Haematological and Serum Parameters

Dietary TM meal inclusion did not influence ($P > 0.05$) the haematological and serum biochemical traits of the animals (Table 3).

Carcass Traits

Dietary TM meal inclusion did not affect ($P > 0.05$) the carcass traits of the broiler chickens (Table 4).

Table 4. Effect of the dietary TM larvae meal inclusion on the carcass traits of the male broiler chickens.¹

Variable	Dietary treatments ²				SEM	P ³	
	C	TM5	TM10	TM15		Linear	Quadratic
Live weight (LW) (g)	3,392	3,572	3,427	3,323	53.17	0.47	0.20
Carcass weight (g)	2,384	2,489	2,403	2,328	41.34	0.51	0.31
Carcass weight (% LW)	70.3	69.6	70.1	70.0	0.35	0.93	0.73
Breast (g)	622	656	664	613	16.20	0.91	0.22
Breast (% LW)	18.3	18.3	19.3	18.4	0.26	0.60	0.35
Thigh (g)	743	781	730	725	12.82	0.37	0.42
Thigh (% LW)	21.9	21.8	21.3	21.8	0.21	0.71	0.53
Spleen (g)	3.93	4.00	3.62	4.39	0.20	0.59	0.41
Spleen (% LW)	0.12	0.11	0.11	0.13	0.01	0.37	0.16
Liver (g)	59.4	66.1	57.8	54.0	1.93	0.15	0.16
Liver (% LW)	1.74	1.85	1.68	1.62	0.03	0.06	0.18
Gizzard (g)	68.6	77.6	68.8	65.9	2.02	0.34	0.14
Gizzard (% LW)	2.02	2.17	2.01	1.98	0.04	0.39	0.27
Abdominal fat (g)	40.5	43.3	33.8	43.5	2.25	0.99	0.46
Abdominal fat (% LW)	1.19	1.22	1.00	1.33	0.07	0.75	0.28

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

²Four dietary treatments: C = control; TM5 = 5% inclusion level of *Tenebrio molitor*; TM10 = 10% inclusion level of *Tenebrio molitor*; TM15 = 15% inclusion level of *Tenebrio molitor*.

³Statistical significance: $P < 0.05$.

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

Index	Fixed effect	d.f. ³	F	P ⁴
Vh (mm)	Diet ¹	3	2.194	0.09
	Intestinal segment ²	2	48.132	<0.001
	Diet × Intestinal segment	6	1.195	0.32
Cd (mm)	Diet	3	2.875	0.040
	Intestinal segment	2	1.911	0.15
	Diet × Intestinal segment	6	0.124	0.99
Vh/Cd (mm/mm)	Diet	3	6.039	0.001
	Intestinal segment	2	15.813	<0.001
	Diet × Intestinal segment	6	0.671	0.67

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

²Three intestinal segments: duodenum, jejunum and ileum.

³Degrees of freedom.

⁴Statistical significance: $P < 0.05$.

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

Histomorphological Investigations

The effects of the diet, intestinal segment, and interaction between diet and intestinal segment on the intestinal morphometric indices are summarized in Tables 5 and 6. Vh depended on intestinal segment ($P < 0.001$) and showed a statistical trend for diet ($P = 0.093$). On the contrary, there was no influence of interaction between diet and intestinal segment ($P > 0.05$) on the morphometric index (Table 5). In particular, duodenum and jejunum showed higher Vh ($P < 0.001$) than ileum. Furthermore, lower Vh was found in TM15 animals ($P = 0.046$ and $P = 0.044$, respectively) compared with C and TM5 (Table 6). There was no effect of intestinal segment or interaction between diet and intestinal segment ($P > 0.05$) on Cd, while diet influenced ($P < 0.05$) the morphometric index (Table 5). In particular, TM15 birds showed higher Cd ($P < 0.01$ and $P < 0.05$, respectively) than C and TM5 (Table 6). Vh/Cd

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

Index	Fixed effect	Effect levels	Least square mean ¹	SEM
Vh (mm)	Diet ²	C	2.05 ^a	0.07
		TM5	2.06 ^a	
		TM10	1.91 ^{a,b}	
		TM15	1.87 ^b	
		DU	2.24 ^a	
Cd (mm)	Intestinal segment ³	JE	2.16 ^a	0.06
		IL	1.52 ^b	
		C	0.17 ^a	
		TM5	0.17 ^a	
		TM10	0.18 ^{a,b}	
Vh/Cd (mm/mm)	Diet	TM15	0.19 ^b	0.01
		DU	0.18	
		JE	0.18	
		IL	0.17	
		C	12.56 ^a	
	Intestinal segment	TM5	12.40 ^a	0.47
		TM10	10.66 ^b	
		TM15	9.87 ^b	
		DU	12.70 ^a	
		JE	12.19 ^a	
		IL	9.23 ^b	

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

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

³DU = duodenum; JE = jejunum; IL = ileum.

depended on diet and intestinal segment ($P < 0.01$ and $P < 0.001$, respectively), whereas there was no influence of interaction between diet and intestinal segment ($P > 0.05$) on the morphometric index (Table 5). In particular, TM10 and TM15 animals showed lower Vh/Cd compared with C ($P < 0.05$ and $P < 0.01$, respectively) and TM5 ($P < 0.05$ and $P < 0.01$, respectively). Furthermore, higher Vh/Cd was found in the duodenum and jejunum ($P < 0.001$) than the ileum (Table 6).

Histopathological alterations varied from absent/minimal to severe in spleen, thymus, bursa of Fabricius, and liver for each dietary treatment, while heart and kidney showed no signs of inflammatory and/or degenerative diseases. Dietary TM inclusion did not affect ($P > 0.05$) the histopathological scores in any of the organs.

DISCUSSION

The present study evaluated the effects of dietary full-fat TM larvae meal inclusion on growth performance, haematochemical parameters, carcass traits, intestinal morphology, and histological findings of male broiler chickens.

Almost 50% of Italian male broiler chickens is generally slaughtered in the period from 50 to 60 days, in order to obtain heavy birds (3.4 to 3.6 kg of LW) for the production of cut-up and further processed products (Bianchi et al., 2007). The slaughter age of the present trial is also in agreement with previous studies (Sirri et al., 2016; Soglia et al., 2016).

Growth performance of the broiler chickens of the current study were consistent with the reference values recorded in the local poultry corporation in which the trial was conducted, but the final LW and overall FCR on average resulted -12.6% and $+16.7\%$ lower and higher than the Ross 708 broiler performance objectives (Aviagen, 2014). This could be related to the physical form of the feed, which was distributed in mash instead of the most frequently used crumble or pellet (Jahan et al., 2006). The body weight and feed intake of the birds in the present trial improved with increasing levels of TM meal inclusion, but the feed efficiency resulted impaired. Data regarding the TM meal utilization in poultry are limited and sometimes controversial. Ramos-Elorduy et al. (2002) showed no effects for the growth performance in fast-growing chickens fed sorghum-soybean meal-based diets in which the full-fat TM inclusion level ranged from 5 to 10% in partial substitution of soybean meal and vegetable oil. Similarly, Biasato et al. (2016) observed unaffected growth performance in intermediate-growing chickens fed corn-soybean-gluten meal-based diets in which the TM meal was included at 7.5% in complete substitution of corn gluten meal. On the contrary, Bovera et al. (2015 and 2016) found improved growth performance in fast-growing chickens fed on a corn-soybean meal-based diets in which the 29.6% of soybean meal was completely replaced by TM meal. In the current study, three different increasing levels of TM meal inclusion were tested in a corn-soybean-gluten meal-based diet replacing a portion of the dietary high-protein feedstuff sources (i.e., soybean and gluten meal) and soybean oil. In the present study, the body weight of the birds fed TM diets increased with increasing feed intake. Even if a feed-choice test was not performed, the increase of the average DFI in the birds may suggest an improvement of the diet palatability related to TM meal inclusion level. To the best of the authors' knowledge,

no feed-choice trial has been performed to test the dietary inclusion of insect meal in broiler chickens. However, Cullere et al. (2016) performed a feed-choice test in broiler quails and observed that the birds tended to prefer the diets including *Hermetia illucens* meal. This may be related to the innate behavior of chickens, which naturally consume insects when reared in free-range systems (Zuidhof et al., 2003). However, the increase of the body weight and feed intake resulted in an overall impairment of the feed efficiency in the birds fed TM diets. Different hypotheses can be formulated to explain this negative effect. Firstly, the detrimental effect on FCR may be caused by the chitin content of TM diets, which has been reported to negatively influence the nutrient digestibility of crude protein (Razdan and Pettersson, 1994; Hossain and Blair, 2007) and organic matter (Razdan and Pettersson, 1994). Ravindran and Blair (1993) also pointed out that the chitin of insects is difficult to digest by domestic poultry. All these authors found no effects on growth performance of chickens fed diets supplemented with commercial sources of chitin instead of the whole insect of the current study (Razdan and Pettersson, 1994; Hossain and Blair, 2007), but the chitin form may have influenced the results. Furthermore, the crude protein digestibility of TM meal used in the current trial was moderate (0.60), as reported by De Marco et al. (2015). Moreover, to the best of the authors' knowledge, this is the first study in which broiler chickens were fed diets with TM larvae meal from the hatch to the slaughter age. Indeed, the TM meal administration of previous studies started from 7 (Ramos-Elorduy et al., 2002; Ballitoc and Sun, 2013), 30 (Bovera et al., 2015), and 43 (Biasato et al., 2016) days of age, respectively, with an inclusion rate ranging from 0.5 (Ballitoc and Sun, 2013) to 29.6% (Bovera et al., 2015). Furthermore, in the present study modification in gut morphology has been observed, mainly in group TM15, and will be discussed successively.

The haematological and serum biochemical parameters give information about health status of the birds (Lumej, 2008; Salamano et al., 2010; De Marco et al., 2013). The results of the present trial suggest that dietary TM meal inclusion did not impair these blood parameters, thus confirming the safety of TM utilization in poultry diets, as previously observed by Bovera et al. (2015) and Biasato et al. (2016). Furthermore, all the blood parameters obtained in the present study fell within the physiological ranges (Lumej, 2008).

Dietary TM meal inclusion did not influence the carcass traits of the birds of the present trial. This is in agreement with Bovera et al. (2016) and Biasato et al. (2016), which did not find any differences for the same considered parameters. A similar figure was given by Hwangbo et al. (2009) and Cullere et al. (2016), who did not observe any effects after house-fly maggots and black soldier fly meal inclusion, respectively, in the diet of broiler chickens and quails. On the contrary, Ballitoc and Sun (2013) found improved slaughter, dressed carcass and eviscerated weights in broiler chickens fed TM diets.

Dietary TM meal inclusion partially affected the intestinal morphology of the broiler chickens of the current study. In particular, shorter villi, deeper crypts, and reduced Vh/Cd were observed in the birds fed with 15% level of TM inclusion compared to the animals fed with TM5 and control diets, respectively. The small intestinal morphology, especially regarding crypts and villi of the absorptive epithelium, plays a key role in the final phase of nutrient digestion and assimilation (Wang and Pen, 2008). The physiological gut morphological development appears to be characterized by the identification of long villi and shallow crypts. Indeed, longer villi are generally associated with increased total luminal absorptive area and subsequent satisfactory digestive enzyme action and higher transport of nutrients (Laudadio et al., 2012). Simultaneously, shallower crypts reflect the prolonged survival of villi without the need for renewal (Oliveira et al., 2008), with reduced energy expenditure for this process and consequent enabled growth of other tissues (Miles et al., 2006). On the contrary, lower villus heights and greater crypt depths may lead to poor digestion and less absorption of nutrients and, as a consequence, poor performance of the animals (Qaisrani et al., 2014). Modifications of protein source and dietary structure has already been reported to negatively influence small intestinal morphology in broilers in terms of decreased Vh and increased Cd (Qaisrani et al., 2014). Therefore, the altered morphometric indices observed in male broilers fed with 15% level of TM meal inclusion could be related to the higher level of insects of this diet, thus also suggesting that lower levels may be preferable. This hypothesis seems to be supported by Biasato et al. (2016), which found no modifications of gut morphometric indices in free-range chickens fed diets including a 7.5% inclusion of TM meal. A similar consideration can also be applied for explaining the growth performance observed in the current study. Indeed, the overall FCR increased linearly with increased levels of TM meal, reaching its maximum with the inclusion of 15% of mealworms. The LW in the finisher period also increased quadratically till 5% level of TM meal inclusion, decreasing up to the inclusion of 15% of insects. Furthermore, the alterations of intestinal morphology can also justify the observed growth performance. Indeed, since it is well known that rapid growth of chickens directly depends on morphological and functional integrity of the digestive tract (Wang and Peng, 2008), the relationship between the altered gut morphometric indices and the worsening of the growth performance seems reasonable.

Apart from TM meal utilization, the current study confirms that morphometric indices followed a proximal-decreasing gradient from the duodenum to the ileum, as previously reported (Iji et al., 2001; Forder et al., 2007; Murakami et al., 2007; Biasato et al., 2016). This is related to the intestinal absorption processes, which evolved differently depending on the considered segment. Indeed, the duodenum is the intestinal tract with the fastest cell renewal, and is also the first segment of the small intestine to receive physical, chemical

and hormonal stimuli provoked by the presence of the diet in the lumen (Macari, 1998). Furthermore, the jejunum is an important site for nutrient digestion (Iji et al., 2001).

Dietary TM meal inclusion did not affect the histopathological features of the birds of the present trial, thus suggesting no negative influence on animal health. This is in agreement with Biasato et al. (2016), which did not find any differences in free-range chickens fed TM diets.

In conclusion, the present study suggests that increasing levels of dietary TM meal inclusion in male broiler chickens diets may improve body weight and feed intake, but negatively affect feed efficiency and intestinal morphology. However, no effect on haematochemical parameters, carcass traits, and histological findings are observed. These results advanced the hypothesis that low levels of TM meal inclusion (i.e., 5%) may be more suitable and overall confirm previous data concerning the safety of TM utilization in poultry feed.

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