POULTRYNSECT

D3.7 Report on gut histology and immunology

Deliverable 3.7

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Abbreviations	
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
BSF	Black Soldier Fly
Cd	Crypt Depth
CREA	Creatinine
GALT	Gut-Associated Lymphoid Tissue
GGT	Gamma Glutamil-Transferase
MT	Mucosal Thickness
MuT	Muscular Thickness
TP	Total Protein
Vh	Villus Height
Vh/Cd	Villus Height To Crypt Depth Ratio
VSA	Villus Surface Area

Content

Introduction

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The POULTRYNSECT Work Package 3 ***"Laboratory and Sensorial Analyses"*** aims to evaluate the impact of Black Soldier Fly (BSF) live larvae inclusion as feed ingredient in chicken diet on chicken health and meat quality. This Deliverable (3.7) reports the assessment of bird`s health obtained from the first and second *in vivo* feeding trials performed with Label Naked Neck and Bionda di Saluzzo chickens, respectively.

Besides morphometric and histopathological analyses on samples of liver, spleen and bursa of Fabricius, a Real Time Quantitative PCR analysis to quantify the MUC-2 gene expression levels has been performed on jejunum samples .

Moreover several blood parameters and immune system traits were analysed in serum samples of the sampled chickens. In particular immunology parameters were performed only in the second trial with Bionda di Saluzzo chickens due to the limited quantity of serum available during the first trial with Label Naked Neck chickens.

1. Material and Methods

In the first trial, a total of 240 Label naked neck birds were distributed in four experimental groups according to gender and treatment (10 chickens/pen, 60 birds/treatment):

1. males fed basal organic feed;
2. males fed basal organic feed +10% BSF supplementation;
3. females fed basal organic feed;
4. females fed basal organic feed +10% BSF supplementation.

The birds were fed with the experimental diets from day 20 to day 82 (time of slaughter) as described in D2.2.

In the second trial, a total of 144 Bianca di Saluzzo male chickens were hatched and reared until 39 days at the Avian Conservation Centre of Local Genetic Resources of the University of Turin (north-west of Italy) and then selected for the experiment on the basis of the average body weight. The trial was carried out from the end of May 2022 until the middle of October 2022. The initial weight of the birds was around 300 g.

The birds were allotted into 18 pens, after being selected and distributed in three experimental groups, according to the diet and live BSF larvae supplementation (8 chicken/pen, 48 birds/treatment):

1. birds fed commercial feed;
2. birds fed sustainable feed;
3. birds fed sustainable feed +15% live BSF larvae supplementation.

Feed and water were provided ad libitum (18 % crude protein, 4.1% crude fat for the commercial feed and 18.2% crude protein, 4% crude fat for the sustainable one) (Mangimi Monge di Monge Antonio e C. Snc). The feed composition of the commercial diet and the alternative one were respectively, as it follows, mainly composed by:

1. commercial diet: corn meal, soybean meal, soybean oil
2. sustainable diet: corn meal, corn gluten, field bean, pea protein, sunflower flour, barley flour.

1.1 Histopathological investigations

At slaughter, samples (approximately 5 cm in length) of duodenum (loop of the duodenum), jejunum (tract before Meckel's diverticulum) and ileum (tract before the ileocolic junction) were excised and flushed with 0.9% saline to remove all the content. Moreover, samples of liver, spleen and bursa of Fabricius were also collected. All the organs were fixed in 10% buffered formalin solution for morphometric and histopathological analyses, following the procedures previously described by **Colombino et al. (2023)**. Briefly, the evaluated morphometric indices were the villus height (Vh), the crypt depth (Cd), mucosal thickness (MT) and muscular thickness (MuT). Thus, the villus height to crypt depth (Vh/Cd) ratio and the villus surface area (VSA) were then calculated. The morphometric analyses were performed on 10 well-oriented and intact villi and 10 crypts chosen from each gut segment (**Qaisrani et al., 2014**). Moreover, MT and MuT were measured on 3 standardized points for each gut segment. Moreover, all the sampled organs were submitted to a semiquantitative histopathological evaluation using the

following score: absent (score = 0), mild (score = 1), moderate (score = 2), and severe (score = 3). Particularly, hepatocyte degeneration and lymphoid tissue activation was evaluated in the liver while follicular depletion was considered in the bursa of Fabricius. Regarding gut histopathological findings, they were separately assessed for mucosa (inflammatory infiltrates) and submucosa (inflammatory infiltrates and Gut-Associated Lymphoid Tissue [GALT] activation) for each segment. Thus, they were combined to obtain the total gut score

1.2 Real Time Quantitative PCR (rt-qPCR)

At slaughter, jejunum from 15 birds/treatment was aseptically collected, placed 24 h in RNAlater (Sigma-Aldrich, MO, USA) at 4 °C and then stored at −80 °C until further analysis. Total RNA was then extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with manufacturer's instructions. The RNA quality of every sample was quantified by Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wiington, DE, USA) and the ratio (OD260:OD280) ranged from 1.7 to 2.1. Afterwards, 2.0 µg of total RNA for each sample was reverse transcribed to cDNA by using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to manufacturer protocol, and the cDNA was stored at −20 °C. rt-qPCR was performed using a 7500 Real Time PCR system (Applied Biosystems, Waltham, MA) in a 20 µL reaction mixture containing 2 µL cDNA, 10 µL of SYBR Green Supermix kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and 0.1 µL of forward and reverse primers (40 mM) of MUC-2 gene (F:5′-ACTCCTCCTTTGTATGCGTGA-3′; R:5′- GTTAACGCTGCATTCAACCTT-3′) . Thermal conditions for performing rt-qPCR were previously reported by **Colombino et al. (2021)**. Relative standard curve method was performed using B-actin and GAPDH as internal control genes to normalize for RNA abundance. Each reaction was run in triplicate. Efficiency curves were performed for each primer set using log10 diluted cDNA in order to obtain efficiency-corrected relative quantification. Amplification efficiency between 90 and 110% was considered good with correlation coefficient (R_2) of 0.99 [**Rebrikov and Trofimov, 2006**].

1.3 Blood analysis and immunology parameters

Blood samples were collected after slaughter, and serum was obtained by centrifugation (Micro 220R, Hettich, Tuttlingen, Germany) at 3500 rpm for 15 minutes and stored at –20°C until analysis. The following blood parameters were determined:
triglycerides, total cholesterol, total protein (TP), alanine aminotransferase (GPT/ALT), aspartate aminotransferase (GOT/AST), creatinine (CREA), gamma glutamil-transferase (GGT). Moreover phosphorus, chlorine, calcium and magnesium concentrations were determined using an automatic chemistry analyzer (BT 1500 vet – Futurlab).
In the second trial, as far the immunology parameters are concerned, the concentrations of the selected serum parameters immunoglobulin M (IgM), immunoglobulin A (IgA), immunoglobulin G (IgG) and interleukin-6 (IL-6) were assayed using species-specific commercial ELISA kits for chicken (Biomatik, Cambridge, Ontario, Canada), the analyses were done according to the manufacturer's instructions.

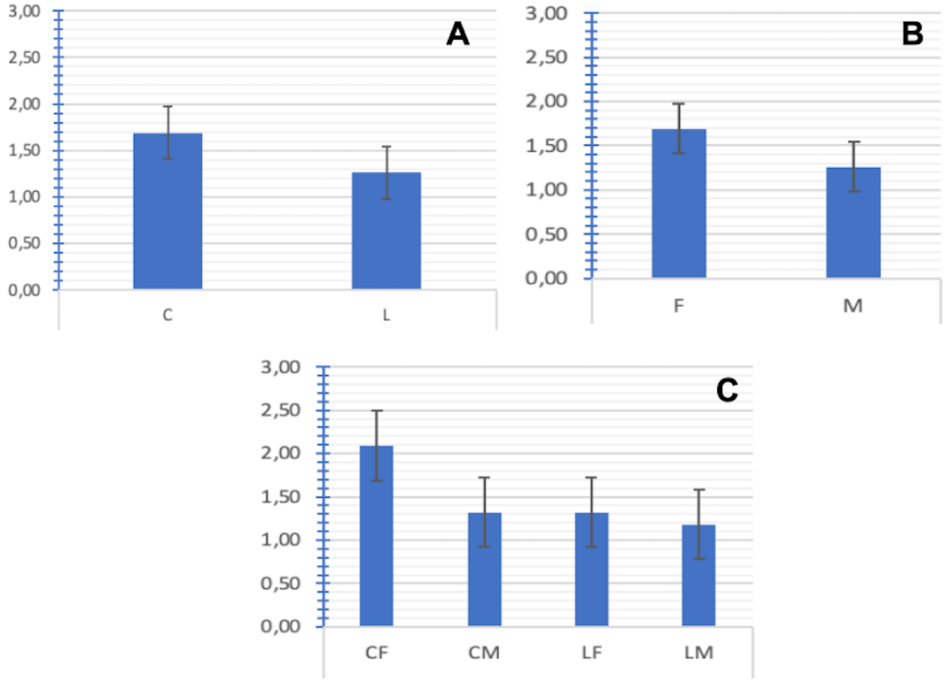
2. Preliminary results and discussion

2.1 Histomorphometric investigations

In the first trial, the results of morphometrical evaluation showed that Vh, VSA and MT depended on sex, being greater in males than in females (P<0.05). Also, Vw was influenced by the interaction diet x sex, being greater in Control Male than in Control Female (P=0.016). Apart from Cd, all the evaluated morphometric indices also depended on gut segment (P<0.001).
Regarding the histopathological scores, diet and the interaction diet x sex did not influence the severity of the histopathological lesions in duodenum, jejunum, ileum, spleen and bursa of Fabricius. However, liver degeneration was influenced by sex, being higher in females than in males (P=0.022)
In the second trial, the results of the histopathology of the organs showed that overall, no differences were observed between the three experimental treatments in duodenum inflammation, jejunum inflammation, ileal inflammation, liver inflammation, liver degradation, spleen hyperplasia and spleen depletion.

2.2 Real Time Quantitative PCR (rt-qPCR)

In the first trial, MUC-2 transcription levels remained unchanged with no significant effect of diet, sex or interaction between them (P>0.05) (Figure 1).
Figure 1 Effect of diet (A), sex (B) and the interaction diet x sex (C) on MUC-2 transcription levels.



2.3 Blood analysis

In the first trial, the results of the live provision of BSFL did not impair most of the hematological traits or serum proteins and lipids, serum minerals, or liver and renal enzymes. As reported in Table 1, Regarding the serum lipids, the triglycerides tended to be more abundant in the F than in the M (P = 0.061), while cholesterol tended to be lower in the treated groups than in the C groups (P = 0.091). As for the serum minerals and the liver and renal enzymes, the live larvae supplementation only influenced the GGT (U/I), which was lower in the treated groups than in the C groups (P < 0.05) (Table 2). No significant effects were observed for the other blood parameters for either the fixed factors (gender and diet) or for the interaction between the gender and diet (P < 0.05).

Table 1. The hematological traits and serum proteins and lipids of the male and female Label Rouge Naked Neck birds (see the reference **Bongiorno et al. 2022**)

Item	Diet (D)		SEM	Gender (G)		SEM	*P*-value		
	BSFL	Control		Male	Female		D	G	D x G
Erythrocytes, 10^6, cell/µL	2.32	2.22	0.23	2.49	2.07	0.23	0.770	0.191	0.941
Leukocytes, 10^3, cell/µL	31.1	23.9	2.26	26.7	27.2	2.24	0.023	0.745	0.955
Heterophils, %	47.3	42.1	2.90	47.2	42.2	2.90	0.202	0.225	0.976
Lymphocytes, %	48.1	52.8	2.65	48.2	52.8	2.65	0.212	0.218	0.955
Eosinophils, %	1.40	1.55	0.18	1.17	1.85	0.19	0.541	0.008	0.385
Monocytes, %	1.63	3.06	3.33	2.85	1.75	0.32	0.002	0.016	0.373
Basophils, %	2.90	2.67	0.32	2.54	3.05	0.32	0.617	0.269	0.495
Serum proteins and lipids									
Total protein, g/dL	4.61	4.73	0.11	4.70	4.65	0.11	0.405	0.743	0.747
Cholesterol, mg/dL	107	119	5.14	116	109	5.14	0.091	0.345	0.520
Triglycerides, mg/dL	112	107	8.54	98.9	121	8.54	0.157	0.061	0.177

BSFL, black soldier fly larvae; SEM, standard error of the mean.

Table 2. The hematological traits and serum proteins and lipids of the male and female Label Rouge Naked Neck birds (see the reference **Bongiorno et al. 2022**)

Item	Diet (D)		SEM	Gender (G)		SEM	*P*-value		
	BSFL	Control		Male	Female		D	G	D x G
Liver function									
ALT, U/l	9.95	11.7	1.03	11.08	10.6	1.03	0.220	0.755	0.383
AST, U/l	167	145	10.27	160	152	10.27	0.134	0.606	0.079
GGT, U/l	22.8	26.9	1.13	26.1	23.6	1.13	0.011	0.114	0.792
Renal function									
Creatinine, mg/dL	0.46	0.47	0.03	0.49	0.44	0.03	0.811	0.148	0.561
Uric acid, mg/dL	8.94	8.95	0.59	8.81	9.07	0.59	0.986	0.752	0.936
Minerals									
P, mg/dL	8.97	9.14	0.31	8.85	9.26	0.31	0.695	0.349	0.294
Fe, µg/dL	101	94.2	17.76	85.5	109	17.76	0.785	0.336	0.300
Mg, mg/dL	1.22	1.10	0.23	1.04	1.28	0.23	0.696	0.450	0.796

BSFL, black soldier fly larvae; SEM, standard error of the mean; ALT, alanine-aminotransferase; AST, aspartate-aminotransferase; GGT, gamma-glutamyl transferase; CRE, creatinine; P, phosphorous; Fe, iron; Mg, magnesium.

In the second trial, the results showed that overall, the provision of live-BSFL had no impact on most of the hematological traits, serum proteins and lipids, serum minerals, as well as liver and renal enzymes. Cholesterol, triglycerides, and chlorine values were affected by age only, at 174 compared to 147 days of age they presented +18.7%, +27.2%

and +6.8%, respectively ($P < 0.05$). Furthermore, GGT and AST values were also affected only by the age of the birds, with a +14% and +10.4% higher value at 174 days of age compared with 147 days, respectively ($P < 0.05$).

As far as the immunology parameters is concerned, neither Immunoglobulins (IgA, IgG and IgM) nor the interleukin (IL-6) levels were affected by diet and/or age effect. The only parameter that showed a tendency to significativity is related to the age effect for the IgA ($P = 0.057$).

References

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