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Inclusion of *Hermetia illucens* larvae reared on fish offal to the diet of broiler quails: Effect on immunity and caecal microbial populations

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Abstract: *Hermetia illucens* (black soldier fly, BSF) larvae meal has shown to be a good protein source in monogastric animal diets, but published data regarding its immunomodulatory properties is limited. For this purpose, a study has been conducted to evaluate the effects of larvae meal on selected immune parameters and caecal bacterial counts of broiler quails. *Hermetia illucens* larvae were reared on two substrates (100% chicken feed, BSF-M, or 50% chicken feed + 50% fish offal, BSF-F) in order to manipulate the fatty acid profile of larvae meal by increasing the long-chain omega-3 (n-3) polyunsaturated fatty acids which are known for their immunomodulatory properties. For immunological purposes, a total of 60 birds were randomly allocated to three dietary treatment groups (n = 20/treatment): control quail diet (CON), or quail diets including 10% of BSF-F or BSF-M larvae meal. Blood was collected 27 and 37 days after the trial commenced to determine the humoral immune response, serum lysozyme concentrations, serum bactericidal activity and protein fractions of the serum. The dietary inclusion of BSF-M meal resulted in an increased secondary humoral immune response compared to the CON treatment group ($P < 0.01$). Quails in the BSF-F treatment had significantly higher serum lysozyme activity compared to quails in the CON and BSF-M treatment group. Both larvae meals significantly increased cell-mediated immunity on day 37 ($P < 0.001$) but they had no effect on serum bactericidal activity. Both larvae meal sources resulted in higher α2-globulin levels on both sampling days, whereas γ-globulin levels were significantly lower in quails with the BSF-F treatment on day 27. Dietary treatments had no significant effect on caecal bacterial counts. In conclusion, the present study showed that the larvae meal had immunostimulatory effects in quails, but the rearing substrate of larvae strongly influenced the immunostimulatory properties of the larvae meal.

Keywords: insect meal; black soldier fly; humoral immunity; cellular immunity; lysozyme; bactericidal activity; serum protein

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The increase of soybean meal and fish meal costs due to the increase of global food and feed demand has resulted in the exploration of new, sustainable protein sources for the monogastric animal feed industry. With the goal to reduce the impact that animal protein production has on the environment, the use of fly larvae meal as an alternative and sustainable protein source in animal feed has gained attention in recent years. The protein and fat content of BSF larvae meal on a dry matter (DM) basis can range from 40% to 52% and 15% to 35%, respectively, depending on the substrate they were reared on (Makkar et al. 2014; Cullere et al. 2016; Spranghers et al. 2017). The black soldier fly larvae meal has a high calcium (5–8% DM) and phosphorus (0.6–1.5%) content and has an amino acid composition similar to soybean meal (Makkar et al. 2014). The majority of studies showed larvae meal to be a good substitute for the conventional protein sources in monogastric diets, resulting in production parameters similar to those of animals fed soybean meal (Cullere et al. 2016). Even though vast amounts of research have been done on insect meal as a protein source, its potential animal health benefits are still unclear. Extracts from housefly (*Musca domestica*) larvae have been proved to exhibit *in vitro* antibacterial, antiviral, and immunomodulatory activity (Meylaers et al. 2004; Ai et al. 2013). It is therefore hypothesised that larvae meal may exhibit similar antimicrobial and immunomodulatory properties when ingested by animals.

The fat content of black soldier fly meal is usually high, but extremely variable, being largely dependent on their rearing substrate and developmental stage (Spranghers et al. 2017). Long-chain unsaturated fatty acids are present in the tissue of several insect species, but the unsaturated fatty acid levels in *H. illucens* larvae meal are low compared to cricket, mealworm and housefly larvae meal (Makkar et al. 2014). However, it is possible to alter the fatty acid composition of *H. illucens* larvae by means of their diet.

For example, St-Hilaire et al. (2007) managed to favourably increase the omega-3 (n-3) fatty acid concentration in *H. illucens* larvae from 0.23% to 2.99% by including 50% fish offal to the cow manure substrate. Dietary n-3 polyunsaturated fatty acids (PUFA's) were proved to enhance the antibody response in quails (Ebeid et al. 2011) and to exhibit several health benefits in other animals (Turchini et al. 2012). Dietary fatty acids and the balance of particular fatty acids, especially the ratio between n-3 and n-6 PUFA's, possess strong immunomodulatory capabilities (Calder 2011). Dietary PUFA's are important building blocks for the cell membrane synthesis and the cell membrane lipids in return provide the substrate for the synthesis of communication molecules involved in the immune system. Therefore, dietary PUFA's indirectly determine the type of immune response that follows the cell damage. Fish oils rich in n-3 PUFA's enhanced antibody production when added to chicken diets (Fritsche et al. 1991). In addition, the increased n-6 : n-3 ratio could decrease mammalian T-lymphocyte proliferation and natural killer cell activity (Baker et al. 1981).

Commercial poultry are vulnerable to stress-related immunosuppression, resulting in vaccination failure, morbidity and possible mortality in flocks. Therefore, the poultry industry could benefit greatly from the use of dietary immunostimulants. If larvae meal still possesses the antibacterial, antiviral and immunomodulatory abilities exhibited in larvae extracts, and the elevated n-3 PUFA concentration in larvae meal exhibits immunostimulatory effects comparable to reported results regarding n-3 levels in animal diets, feeding BSF larvae meal or n-3 enriched BSF larvae meal to monogastric animals may alleviate the need for antibiotics. Based on the above-mentioned considerations, the aim of the study was to determine the effects of larvae meal grown on two different substrates (fish offal or chicken feed) on selected quail immune parameters and caecal bacterial counts.

MATERIAL AND METHODS

Insect rearing

Black soldier fly larvae used in the trial were reared on two different substrates. To produce the BSF-M larvae meal, freshly hatched BSF neonatal larvae were reared on a commercial chicken layer mash (crude protein = 130 g/kg; fat = 25 g/kg; fibre = 70 g/kg; moisture = 120 g/kg; Ca = 35 g/kg; P = 5 g/kg; lysine = 5 g/kg), soaked in hot water (water/mash = 1.6 : 1) for 16 hours. For the BSF-F larvae meal, a substrate consisting of a mixture of 50% soaked layer mash and 50% minced fish offal was fed to the larvae. The same batch of soaked

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chicken feed was used for the two larval diets. Larvae were reared in an environmentally controlled room (temperature of 27 ± 1 °C and relative humidity of 65 ± 5%) and harvested after 16 days, before reaching the 6th instar (prepupae). In order to kill the larvae and inhibit tyrosinase activation and autolysis, the larvae were placed in boiling water for 1 minute. Subsequently, the larvae were rinsed to remove all the debris, dried in a commercial oven for 16 h at 65 °C and finely ground afterwards. Chemical composition and amino acid profile are presented in Table 1.

Animals and diets

To determine the immunomodulatory and antimicrobial effects of the dietary treatments, a total of 90 ten-day-old broiler quails were randomly selected from a commercial quail farm in the Vicenza province (Italy). The quails were housed in battery cages in an environmentally controlled room (±26 °C and ±72% relative humidity) on the farm during the trial period of 37 days. The trial ran in conjunction with performance trial as presented in Woods et al. (2019), and with meat quality trial as presented in Cullere et al. (2019). A total of 60 birds (20 birds/treatment) for immunological purposes, and 30 birds (10 birds/treatment) for caecal bacterial counts were randomly allocated to one of the three dietary treatments (Table 2). The control diet (CON) was a standard grower diet formulated according to the nutrient requirements for broiler quails. To formulate the BSF-M and BSF-F treatment diets, either 10% BSF-M larvae meal or 10% BSF-F larvae meal were added to the CON diets through partly replacing conventional protein/fat sources, while maintaining an isonitrogenous and isocaloric diet (Table 2). The trial protocol was approved by the veterinary authority and carried out according to the article '2, DL 4 March 2014, No. 26' of the Official Journal of the Italian Republic (http://www.gazzettaufficiale.it/eli/id/2014/03/14/14G00036/sg), implementing the EC Directive 86/60963/2010 EU regarding the protection of animals used for experimental and other scientific purposes.

Table 1. Chemical composition and amino acid concentration (g/kg as fed) of the two batches of *Hermetia illucens* larvae meal

	BSF-M larvae meal	BSF-F larvae meal
Dry matter (DM)	934.9	940.1
Crude protein	336.6	343.1
Crude fat	342.0	366.9
Crude fibre	55.4	51.3
Chitin	24.8	24.2
Ash	135.2	99.2
Ca	43.4	29.1
P	5.91	5.75
Gross energy (MJ/kg)[1]	22.63	23.36
Arginine	19.1	37.9
Histidine	13.4	21.2
Isoleucine	13.1	15.3
Leucine	20.4	21.8
Lysine	18.8	22.4
Methionine	5.0	6.3
Phenylalanine	13.9	21.4
Threonine	13.3	16.5
Valine	17.3	21.2

BSF-M and BSF-F = control diet supplemented with dried *H. illucens* larvae reared on layer mash (BSF-M) or on 50 : 50 layer mash and fish offal (BSF-F)

[1]Analysed with an adiabatic bomb calorimeter (ISO 1998)

Immunisation and blood collection

On day 20 and 30 of feeding the treatment diets, quails were injected intramuscularly with 0.25 ml of 10% Porcine Red Blood Cells (PRBC; Innovative Research Inc., Southfield, MI, USA). Blood was collected from the brachial vein 7 days after each injection to determine the primary and secondary antibody response. Blood was transferred into tubes containing a clot activator. Serum was separated by centrifugation at 1 800 *g* for 10 min at 4 °C and stored at –20 °C until further analysis.

Haemagglutination assay

Serum samples used for the haemagglutination assay were placed in a water bath set at 56 °C for 30 min to inactivate the complement. A volume of 50 µl of phosphate-buffered saline (PBS) pH 7.2 was added to each well of a 96-well U-shaped bottom microplate. Subsequently, 50 µl of serum was

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Table 2. Ingredient composition and analysed nutrient composition of the experimental diets

	Treatment diets		
	CON	BSF-M	BSF-F
Ingredients (g/kg)			
Maize (fine)	435.6	450.5	434.8
Soybean meal	460.4	377.0	373.5
Dried *Hermetia illucens* larvae (BSF)	0.0	100.0	100.0
Whole wheat (fine)	23.5	42.5	61.7
Calcium carbonate	21.5	20.0	20.0
NaCl	2.7	2.7	2.7
L-lysine	0.5	0.5	0.5
DL-methionine	1.8	1.8	1.8
Vitamin-mineral premix[1]	5.0	5.0	5.0
Soybean oil	49.0	0.0	0.0
Nutritional value (analysed)			
Gross energy (MJ/kg)	17.4	17.9	17.8
Crude protein (g/kg)	243.3	238.9	239.8
Nutritionally important PUFA's (analysed %)			
C18:2n-6 (LA)	51.3	25.1	18.8
C18:3n-3 (ALA)	4.37	1.16	1.35
C20:4n-6 (ARA)	0.06	0.06	0.09
C20:5n-3 (EPA)	0.08	0.05	0.58
C22:6n-3 (DHA)	0.00	0.00	0.10

ALA = α-linolenic acid; ARA = arachidonic acid; BSF-M and BSF-F = control diet supplemented with dried *H. illucens* larvae reared on layer mash (BSF-M) or on 50 : 50 layer mash and fish offal (BSF-F); CON = control diet; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; LA= linoleic acid

[1]Vitamin and mineral premix provided the following per kg of diet: vitamin A, 20 000 IU; vitamin D3, 6 000 IU; vitamin E (α-tocopherol acetate), 90 mg; vitamin K3, 7 mg; vitamin B1, 3.5 mg; vitamin B2, 16 mg; niacinamide, 100 mg; vitamin B6, 8 mg; vitamin B12, 0.04 mg; biotin, 0.4 mg; folic acid, 2.5 mg; Ca-pantothenate, 27.78 mg; Fe, 80 mg; Mn, 200 mg; Cu, 50 mg; Zn, 200 mg; Ca-iodate, 2 mg/kg; Se, 0.4 mg; E 1 604 Endo 14, 2 200 IU; Endo 1, 3 000 FTU; sepiolite, 175 mg

added to the first well of each row and each sample was twofold serially diluted from 1 : 2 to 1 : 2 048 into the remaining wells of the row. 50 µl of 1% PRBC suspension was added to each well. Total antibody titres (HA titre) were read after 30 min incubation at 37 °C. Titres were expressed as Log_2 of the reciprocal of the highest dilution giving visible agglutination and the activity of total haemagglutinating antibodies that consists of immunoglobulin M and immunoglobulin G antibodies was measured (Khatibjoo et al. 2011).

Serum bactericidal activity (SBA)

To determine the bactericidal activity of the serum, a turbidimetric assay in microtitre format was performed. Briefly, a non-pathogenic strain of *Escherichia coli* was suspended in 15 ml Brain Heart Infusion broth (BHI; Oxoid™, Thermo Fisher Scientific Inc., Hampshire, UK), incubated at 37 °C until optical density at 590 nm doubled; an indication that the log phase of growth was reached. Sterile saline solution was used to dilute the bacteria to a 1 : 100 dilution. Subsequently, 50 µl of serum (in duplicate) were distributed in the wells, followed by 50 µl veronal buffer, 100 µl of BHI broth and 10 µl of the diluted bacterial suspension. Controls of bacterial growth and sterility were set up without serum. Plates were covered and incubated in a humidified box at 37 °C for 18 hours. The optical density (OD) of the samples in the microtitre plates was read spectrophotometrically in an ELISA reader (Multiskan FC microplate reader; Thermo Fisher Scientific, Waltham, MA, USA) at 690 nm with the blank set on the sterility control. SBA percentage was derived by the following equation:

$$\%\,SBA = (OD\ test\ sample/OD\ growth\ control) \times 100 \quad (1)$$

where:
SBA – serum bactericidal activity;
OD – optical density.

Serum lysozyme concentration

Lysozyme concentration was determined by measuring the lysed areas around the serum samples placed in wells in agar containing *Micrococcus lysodeikticus*. To prepare the plates, 1 g agarose (type II medium EEO, A-6877; Darmstadt, Sigma-Aldrich, Germany) was added to 100 ml sodium phosphate

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buffer (pH 6.3) and boiled until a homogenous solution was obtained. The medium was maintained at a temperature of 60 °C in a warm bath with constant stirring. A bacterial suspension was prepared by adding 0.1 g *M. lysodeikticus* (M-3770; Sigma-Aldrich, Darmstadt, Germany) to 2 ml sodium phosphate buffer. Subsequently 200 µl bacterial suspension was added to each 100 ml agar solution. The homogeneous bacterial agar solutions (50 ml) were poured into 10 cm square petri dishes and wells of 2 mm diameter, spaces 2 cm from each other were made in the agar with a thin-walled brass tube connected to a vacuum pump. Wells were filled with 20 µl serum (in duplicate). Standard dilutions (512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5 µg/ml) of purified egg white lysozyme (62971; Sigma-Aldrich, Darmstadt, Germany) in PBS (pH 6.3) were run with the test sample. The plates were incubated in a humidified box at 37 °C for 18 hours. The diameter of the lysed area around the wells filled with serum or lysozyme standards was measured with a calliper. The lysozyme concentration (µg/ml) of the serum samples was proportional to the diameter of lysed areas and was determined from a semi-logarithmic curve created from the purified lysozyme standards.

Wing web thickness index

The lymphoproliferative response to phytohaemagglutinin (PHA-P), an indicator of T-cell mediated immune responsiveness in animals, was assessed 20 days after the commencement of treatment diets using the procedures of Corrier and DeLoach (1990).

Briefly, a dose of 0.1 ml of 1 mg phytohaemagglutinin (PHA-P, L8754; Sigma Aldrich, St. Louis, MO, USA) dissolved in 100 µl PBS was injected intradermally into the left wing web of the birds while the right wing web received a control injection of 0.1 ml sterile PBS alone. The thickness of each wing web was measured immediately before and 24 h post injection with a thickness meter to the nearest 0.01 mm. The wing web swelling reactions to PHA-P were calculated using the following swelling index:

$$\text{Index} = (\text{thickness post-PHA-P injection} - \text{thickness pre-PHA-P injection}) - (\text{thickness post-PBS injection} - \text{thickness pre-PBS injection}) \quad (2)$$

Bacterial enumeration from caecal digesta

After receiving the dietary treatment for 20 days, 30 birds (n = 10/treatment) were killed by means of cervical dislocation. Subsequently, carcasses were opened, the caeca were removed aseptically and the contents of the caeca were placed in a sterile test tube, weighed and diluted 1 : 10 with sterile saline solution. The digesta homogenate was serially diluted from 10^{-1} to 10^{-5}. Dilutions were plated in duplicate on selective agar media for the enumeration of target bacteria groups. In particular, total viable counts, total coliforms, *Staphylococcus* spp., *Micrococcus* spp., *Lactobacillus* spp. and *Aeromonas* spp. + *Pseudomonas* spp. were enumerated using Plate count agar (PCA), MacConkey agar (MCC), Baird-Parker agar (BP), Mannitol salt agar (MSA), De Man, Rogosa & Sharpe agar (MRS) and Glutamate starch phenol red agar (GSP), respectively.

All agar media were purchased from Oxoid™ – Thermo Scientific™ (Thermo Fisher Scientific Inc., Hampshire, UK). The plates were incubated at 37 °C for 24 h, with the exception of MRS agar (at 30 °C in a 5% CO_2 atmosphere for 72 h) and GSP agar (at 25 °C for 48 hours).

After the incubation period, the colonies were counted.

Serum protein fractions

Serum biochemistry was determined by means of a Hitachi 912 automatic analyser (Roche Diagnostics GmbH, Mannheim, Germany). Serum protein separations were made using the P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Inc., Fullerton, CA, USA).

Statistical analysis

All the parameters were subjected to one-way ANOVA with experimental diets as fixed effect, following the GLM procedure of the SAS v9.1.3 statistical analysis software for Windows (SAS Institute 2008).

Differences were considered significant when $P < 0.05$. When significant differences occurred, treatment mean differences were identified by pairwise comparison using the Bonferroni test.

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RESULTS AND DISCUSSION

Humoral antibody response, lysozyme activity, bactericidal activity and cellular immune response

For trial purposes, BSF-F larvae meal was enriched with two long-chain n-3 PUFA's, eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). The CON diet had higher levels of linoleic acid (LA, C18:2n-6) compared to the larvae meal diets (Table 2). Linoleic acid is usually converted into arachidonic acid (AA, 20:4n-6) (Calder 2011). Arachidonic acid and long-chain n-3 PUFA's compete for incorporation into cell membrane phospholipids (Calder 2007). Both AA and DHA are precursors of eicosanoids by means of cyclooxygenase and lipoxygenase. However, the eicosanoids derived from AA in membranes, including thromboxanes and prostaglandin E2 (PGE2), are more pro-inflammatory than those derived from DHA (Calder 2007). Prostaglandin E2 is also well known for its immunosuppressive effects. Therefore, the levels of DHA or AA deposited in the cells, as a result of the long-chain n-3/n-6 ratio, should have considerable effects on the function of the immune system. A weak primary humoral immune response was observed for all the treatments with no significant differences in antibody production (Table 3). After the second immunisation with PRBC, HA titres were significantly higher for the BSF-M treatment group compared to the CON treatment group, with titres for the BSF-F treatment group being intermediate (P = 0.006). Acquired immunity has two arms, namely humoral immunity and cellular immunity. Humoral immunity is mediated by serum antibodies, secreted by B-cells after binding with antigens. The quails in this study were challenged twice with PRBC, a non-pathogenic antigen, to evoke a primary and secondary humoral immune response, measured by a haemagglutination assay. A relatively small primary humoral response was detected, with no differences between the treatment groups. However, after the second PRBC immunization, the secondary response of the BSF-M group was significantly higher compared to the CON treatment group, with the BSF-F group being intermediate (Table 3). Publications regarding the effect of larvae meal on the humoral immune response in animals are scarce. The present study is the first to determine the antibody production ability after the immunization of animals fed larvae meal-containing diets. Although the HA titre for the BSF-F group did not differ significantly from the BSF-M group, a reduction in antibody synthesis occurred, owing to the HA titre similar to the CON group. The reason for the reduced titre is unclear. Considering the important immunomodulatory effects long-chain n-3

Table 3. Effect of dietary inclusion of *Hermetia illucens* larvae meal on the humoral immune response, lysozyme activity, bactericidal activity and cellular immune response measured in Japanese quails (mean ± SE)

	Treatment diets			*P*-values
	CON	BSF-M	BSF-F	
Animals (*n*)	20	20	20	
Haemagglutination titre				
Primary response	0.41 ± 0.24	0.82 ± 0.29	0.77 ± 0.41	0.180
Secondary response	3.5^{b} ± 0.50	5.7^{a} ± 0.45	4.6^{ab} ± 0.37	0.006
Lysozyme activity (mg/ml)				
Day 27	150 ± 20	165 ± 24	239 ± 53	0.173
Day 37	91^{b} ± 9	125^{b} ± 20	206^{a} ± 23	< 0.001
Bactericidal activity (%)				
Day 27	68 ± 1.45	62 ± 3.09	58 ± 4.33	0.160
Day 37	51 ± 3.84	49 ± 5.80	51 ± 3.61	0.954
Wing web swelling index (mm)	0.27^{c} ± 0.05	0.66^{b} ± 0.09	1.03^{a} ± 0.07	< 0.001

BSF-M and BSF-F = control diet supplemented with dried *H. illucens* larvae reared on layer mash (BSF-M) or on 50 : 50 layer mash and fish offal (BSF-F); CON = control diet

[a,b,c]Means within rows with different superscripts differ significantly (P < 0.05)

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PUFA's exert in animals, their levels in BSF-F diet might be the reason for this result. However, previous studies that examined the effect of n-3/n-6 ratios on the humoral immune response by adding fish oil to poultry diets were either unsuccessful in changing the HA titre, or a positive result was observed (Khatibjoo et al. 2011). Therefore, several unknown factors in the BSF-F meal could have contributed to this phenomenon.

On day 27 of the trial, no differences in serum lysozyme activities were observed between treatments. However, on day 37, quails in the BSF-F treatment group had significantly higher lysozyme activity compared to chicks receiving the CON or BSF-M diet. Lysozymes are glycoside hydrolases secreted by phagocytes and are involved in the innate immune response of animals, exerting a synergic action with the complement system and the humoral immune response. Higher levels of lysozyme activity in the sera of the BSF-F group could be an indication that larvae meal reared on fish has certain properties to stimulate the activation of these phagocytes, enhancing the antibacterial defence mechanisms of quails. The mechanism might be the decreased production of some metabolites like PGE2 due to the increased long-chain n-3 PUFA's and the shift in n-3/n-6, which in turn transforms the membrane composition of immune cells (Calder 2007). A negative correlation exists between the level of long-chain n-3 fatty acids in the diet and the production of PGE2 (Guo et al. 2004), whereas n-6 fatty acids enhance the synthesis of PGE2 of peripheral blood leucocytes (Konieczka et al. 2017). More importantly, PGE2 negatively correlates with lysozyme activity (Guo et al. 2004). Furthermore, in the current research, DHA was detected only in the BSF-F feed (Table 2). Protectin D1, a mediator from DHA, enhances the phagocytic activity of macrophages (Schwab et al. 2007), the main producers of lysozyme in blood, hence partly explaining the increased lysozyme concentration in serum from quails in the BSF-F treatment group. Even though no similar studies could be found on the effect of larvae meal or n-3 enriched larvae meal on serum lysozyme activity in poultry, previous research indicated an enhancement in serum lysozyme when laying hens received n-3 enriched diets (Guo et al. 2004).

As mentioned above, lysozymes exert a synergistic action with the complement, a system composed of serum proteins that react against pathogens through a molecular cascade, resulting in microbial lysis. Lysozymes, together with the complement and antimicrobial peptides (AMP's) in blood, are all responsible for the bactericidal properties of the serum (Riera Romo et al. 2016). Even though dietary treatments in this study resulted in differences in serum lysozyme activity against gram-positive bacteria, it should be noted that no differences were observed when taking into account the total serum bactericidal activity against the gram-negative bacteria *E. coli.*

In the present study, the inclusion of insect meal in the diets of quails enhanced cellular immune responses when considering the swelling response to PHA-P, with the greatest response in quails with the BSF-F treatment, followed by BSF-M quails, and lastly the quails with the CON treatment exhibiting the lowest response ($P < 0.001$).

The induced swelling of the wing web 24 h after mitogen injection can be an indication of enhanced lymphoproliferative ability of the immune system. Phytohaemagglutinin (PHA-P) is a T-cell mitogen used to measure the T-lymphocyte function. Injecting PHA-P into the skin of animals stimulates T-cell proliferation, differentiation, and cytokine production, causing an influx of basophils and other leucocytes into the injection site, resulting in the skin swelling (Corrier and DeLoach 1990). In addition, results in this study demonstrated that cellular immune responses can be even further enhanced when larvae meal is enriched with long-chain n-3 fatty acids. These results are in agreement with Korver and Klasing (1997) and Agazzi et al. (2004), who showed that the inclusion of omega-3 in animal diets by means of fish oil increases the swelling response. Granted that PHA-P stimulates T-cell proliferation and PGE2 suppresses it (Baker et al. 1981), this result can be expected. Contrary to these observations, Khatibjoo et al. (2011) reported a smaller swelling response in animals when dietary n-3 : n-6 ratios were low and they hypothesized it to be due to the reduced levels of AA and pro-inflammatory AA-derived eicosanoids deposited into the cells. However, as reported by Korver and Klasing (1997), the inclusion of fish oil in the diets of broilers improves the PHA-P response, but decreases the inflammatory response indices. Moreover, Konieczka et al. (2017) demonstrated that even though the levels of pro-inflammatory eicosanoids (PGE2 and thromboxane) decreased in the blood of n-3 enriched chickens, basophil and macrophage count still increased after PHA-P injection.

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Serum protein fractions

For serum collected on day 27 (Table 4), no significant differences were observed in total protein, prealbumin, albumin, α1-globulin or β1-globulin concentrations between the three experimental groups. However, the dietary inclusion of any of the BSF larvae meals significantly increased the α2-globulin concentration compared to the CON treatment ($P < 0.05$). Dietary inclusion of BSF-F meal significantly decreased γ-globulin compared to the BSF-M and CON treatment groups on day 27 ($P < 0.05$). Serum protein and protein fractions were overall comparable on day 37. The only exception was α2-globulin, being again higher in the two larvae meal treatment groups when compared to the CON treatment ($P < 0.05$).

Serum proteins have various physiological roles and are therefore a significant indicator of the birds' health condition, including immune and inflammatory responses. Total serum protein in quails receiving the three dietary treatments was similar to the average levels for treatments being 27.4, 30.2 and 27.7 g/l for the CON, BSF-M and BSF-F treatment groups, respectively, which is within the normal concentration of 30–50 g/l for most avian species. A reduction of total serum protein can be an indicator of malnutrition, malabsorption, heavy metal poisoning, intestinal parasitism, prolonged stressed or liver poisoning (Crespo and Shivaprasad 2014). However, in spite of the slight decrease in average serum protein levels in CON and BSF-F quails, resulting in levels lower than the average for avian species, there were no significant differences between the treatments. The low total protein levels are not therefore treatment-related but they are rather due to other environmental factors.

Albumin and the four globulin fractions (α1, α2, β, and γ-globulins) are five of the main fractions of serum protein. Many acute phase proteins can

Table 4. Effect of dietary inclusion of *Hermetia illucens* larvae meal on total serum protein and serum protein fractions measured in Japanese quails (mean ± SE)

	Treatment diets			*P*-values
	CON	BSF-M	BSF-F	
Animals (*n*)	20	20	20	
Day 27				
Total protein (g/l)	27.42 ± 0.70	30.21 ± 4.40	27.67 ± 1.22	0.224
Prealbumin (g/l)	0.45 ± 0.16	0.28 ± 0.04	0.31 ± 0.04	0.252
Albumin (g/l)	11.00 ± 0.72	12.24 ± 0.92	11.85 ± 3.11	0.559
α1-globulin (g/l)	0.83 ± 0.11	0.70 ± 0.08	0.86 ± 0.67	0.526
α2-globulin (g/l)	6.11^{b} ± 0.22	8.02^{a} ± 0.27	7.57^{a} ± 0.25	< 0.001
β1-globulin (g/l)	6.21 ± 0.59	6.04 ± 0.50	5.10 ± 0.44	0.239
γ-globulin (g/l)	2.84^{a} ± 0.39	2.91^{a} ± 0.29	1.97^{b} ± 0.21	0.042
Albumin/globulin	0.69 ± 0.04	0.69 ± 0.04	0.75 ± 0.03	0.338
Day 37				
Total protein (g/l)	31.46 ± 1.76	31.00 ± 2.01	30.69 ± 1.52	0.621
Prealbumin (g/l)	0.62 ± 0.18	0.41 ± 0.12	0.36 ± 0.06	0.374
Albumin (g/l)	12.35 ± 0.95	11.31 ± 0.92	10.44 ± 0.76	0.170
α1-globulin (g/l)	0.86 ± 0.66	0.66 ± 0.48	0.68 ± 0.48	0.284
α2-globulin (g/l)	7.26^{b} ± 0.26	8.62^{a} ± 0.36	8.38^{a} ± 0.32	0.014
β1-globulin (g/l)	7.47 ± 0.68	6.81 ± 0.90	7.20 ± 1.10	0.877
γ-globulin (g/l)	2.50 ± 0.27	3.40 ± 0.35	3.64 ± 0.511	0.203
Albumin/globulin	0.67 ± 0.04	0.59 ± 0.03	0.54 ± 0.04	0.064

BSF-M and BSF-F = control diet supplemented with dried *H. illucens* larvae reared on layer mash (BSF-M) or on 50 : 50 layer mash and fish offal (BSF-F); CON = control diet

[a,b]Means within rows with different superscripts differ significantly ($P < 0.05$)

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be classified under α-globulins. In the current trial, α2-globulin levels were significantly higher in the BSF-M and BSF-F treatment compared to the control treatment for both sampling days. Although an increase in α-globulins or β-globulins can be an indication of inflammation, some proteins like α2-macroglobulin, haptoglobin and ceruloplasmin classified under α2-globulin, can have numerous benefits for the animal. Not only does ceruloplasmin scavenge free radicals (Cray et al. 2009), but also haptoglobin, or its avian counterpart called PIT54, binds to free haemoglobin, preventing oxidative damage and loss of iron. A reduction of free iron limits the availability of iron needed for bacterial growth. For this reason, haptoglobin is believed to exhibit bacteriostatic properties. Lastly, α2-macroglobulin and homologues of this macroglobulin detected in poultry (Starkey and Barrett 1982) inactivate toxins (Bort 1992), remove enzymes released during injury (Cray et al. 2009) and are protease inhibitors capable of inhibiting both endogenous and exogenous proteases (Magor 2001). Proteases secreted by parasites play an important role in parasitic virulence, however α2-macroglobulin can bind these proteases, resulting in their degradation by means of lysosomes (Van Leuven et al. 1978). It should be noted that all the quails in this study appeared healthy, therefore, although α-globulins can be associated with inflammation, and considering the beneficial effects of α2-globulin proteins, the increased α2-globulin levels in the serum of BSF-M and BSF-F quails can be an indication that BSF larvae meal possesses immunostimulatory properties in this regard.

The γ-globulin fraction represents the immunoglobulins (antibodies) in the serum and takes into account the normal immunoglobulin levels together with the increased levels after a humoral immune response to an antigen. Even though the HA titre as a response to PRBC did not differ after the first immunisation, the γ-globulin levels of quails in the CON and BSF-M group were significantly higher compared to those in the BSF-F group. However, after the second immunisation, the γ-globulin concentration in BSF-F quails almost doubled while the responses in the CON and BSF-M treatment groups were relatively small, therefore diminishing the differences observed on day 27. Published data on the effect of fly larvae meal on serum protein in poultry is scarce, however previous research (Bovera et al. 2015) measured total protein, albumin and total globulin levels in chickens fed mealworms. Similarly to the present study, insect meal had no effect on these parameters, but a smaller albumin/globulin ratio in chickens fed insect meal was noted and the improved immune response was attributed to the properties of the chitin content of insects.

Caecal bacterial counts

No differences were noticed between treatments for the microbial composition of the caecal content when considering total viable counts, total coliforms, *Staphylococcus* spp., *Micrococcus* spp., *Aeromonas* spp., *Pseudomonas* spp. or *Lactobacillus* spp. (Table 5). Chitin is believed to serve as a prebiotic in the large intestine of poultry (Bovera et al.

Table 5. Effect of dietary inclusion of *Hermetia illucens* larvae meal on selected microbial count (CFU/g) in the caecal content of Japanese quails (mean ± SE)

	Treatment diets			*P*-values
	CON	BSF-M	BSF-F	
Animals (*n*)	10	10	10	
Total viable count	8.21 ± 0.26	7.30 ± 0.32	7.43 ± 0.36	0.111
Total coliforms	6.54 ± 0.45	6.05 ± 0.45	6.55 ± 0.46	0.670
Staphylococcus spp.	3.06 ± 0.12	3.02 ± 0.33	2.93 ± 0.21	0.931
Micrococcus spp.	4.29 ± 0.36	4.69 ± 0.36	3.91 ± 0.28	0.201
Aeromonas spp.	2.60 ± 0.46	3.07 ± 0.46	3.31 ± 0.48	0.550
Pseudomonas spp.	6.38 ± 0.16	5.61 ± 0.30	5.70 ± 0.30	0.101
Lactobacillus spp.	3.82 ± 0.12	4.14 ± 0.23	3.85 ± 0.15	0.352

BSF-M and BSF-F = control diet supplemented with dried *H. illucens* larvae reared on layer mash (BSF-M) or on 50 : 50 layer mash and fish offal (BSF-F); CON = control diet

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2015). However, Khempaka et al. (2011) reported that purified chitin was incapable of altering the intestinal populations of *Salmonella* spp., *E. coli* or *Lactobacillus* spp. in broilers, whereas the chitin constituent in shrimp meal, represented in the form of a chitin-protein complex, resulted in a favourable shift in the intestinal microflora. Hence because chitin also combines with proteins in the cuticles of insects, a favourable shift in the intestinal microflora can also be expected in poultry consuming insect meal. However, there was no shift in caecal microbial populations when considering the bacteria enumerated in this study. This is consistent with the work published by Cullere et al. (2016). Although this may be true of the few bacterial populations tested in this study, a different outcome may be expected when taking into account the entire microbial ecology of the gastrointestinal tract. By means of 16S rDNA sequencing, Borrelli et al. (2017) showed that *H. illucens* larvae meal in the diets of laying hens can cause a major beneficial shift in the caecal microbiota when taking into account microbial species as well as relative abundance; the insect diet increased the numbers of bacteria capable of degrading chitin and increased the richness of the microbiota, which in turn is usually associated with a good health status.

CONCLUSION

In summary, feeding quails with larvae meal from larvae that were reared on fish offal (with higher levels of n-3 PUFA's) resulted in an improved cell-mediated immunity and an increase in serum lysozyme concentrations. On the other hand, quails receiving larvae meal that were reared on chicken feed had an improved secondary immune response. Dietary treatments did not alter serum bactericidal activity, caecal bacterial counts or most of the serum protein fractions, however both larvae meal sources increased α2-globulin concentrations in the serum. It can be concluded that BSF larvae meal has immunostimulatory effects in broiler quails, although the substrate for larvae rearing influences the immune response by the animal.

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Conflict of interest

The authors declare no conflict of interest.

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