Final Report

Project code: 18-409

Prepared by: Isabelle Ruhnke, Jessica DeSouza Vilela, Manisha Kolakshyapati

Date: 03.04.2020

Black soldier fly in poultry feed

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Researcher Contact Details

Name: Isabelle Ruhnke
Organisation University of New England
Phone: + 61 2 6773 5155
Email iruhnke@une.edu.au
Website: https://www.une.edu.au/staff-profiles/ers/iruhnke

Poultry Hub Australia Contact Details

Poultry Hub Australia

CJ Hawkins Homestead, Ring Road

Armidale NSW 2350

02 6773 1855

poultryhub@une.edu.au

www.poultryhub.org

Project Summary

Project Title	Black soldier fly in poultry feed
Project No.	18-409
Date	Start: 01.10.2018 End: 15.12.2019
Project Leader(s)	Isabelle Ruhnke
Organisation	University of New England
Email	iruhnke@une.edu.au
Project Aim	The aim of the project was to evaluate the effects of including of different levels of black soldier fly (BSF) larvae (5%, 10%, 15% and 20%) in broiler feed and investigate its impact on various parameters including broiler performance, health, nutrient digestibility and meat quality.
Background	Black soldier fly (BSF) larvae are known for their high nutritional value, antimicrobial properties, and the ability of being produced using organic waste, which makes this larva a very sustainable option to be included in livestock feed. However, the impact of maximum inclusion levels in broiler diets on bird performance and meat quality are currently unknown. In addition, investigate of the impact of the maximum inclusion levels of the BSF larvae on broiler's immune system, and the bird's intestinal microbiota was warranted.
Research Outcome	Up to 20%, BSF larvae could be included in balanced broiler diets that were fed when birds aged day 2 until day 42 without compromising the broiler's performance and meat quality. In detail, there was no effect on dry matter, crude protein or ash digestibility during the grower period, but a significant increase in fat digestibility. The impact of the intestinal microbial population was minor where the abundance of *Enterococcus spp* and an increase in *Dehalobacterium* was observed when BSF larvae levels were increased. A negative correlation of the BSF larvae could be observed on intraepithelial and blood lymphocytes, whereas increasing BSF larvae levels decreased these immune parameters linearly. No effect was observed in any of the meat quality parameters except for the fatty acid profile of breast meat, whereas BSF larvae significantly increased the saturated fatty acids, decreased the unsaturated fatty acids, and increased lauric acid and eicosapentaenoic acid.
Impacts and Outcomes	Up to 20% of BSF larvae can be included in balanced broiler diets and fed from day 2 to day 42 without compromising the broiler's performance, nutrient digestibility, intestinal microbiota composition, and meat quality. Further research is required to investigate whether the effects of the BSF larvae on the immune system can have clinical impact e.g. health benefits.

Publications	De Vilela JS, Andronicos N, Hilliar M, Andrew N, Swick R, Ruhnke I (2019) Black soldier fly larvae in broiler diets did not affect performance but decreased cellular immune parameters. In '22nd European Symposium of Poultry Nutrition', 10–13 June 2019, Gdansk, Poland, Proceedings pg. 159. De Vilela JS, Alvarenga TIRC, Hopkins M, Kolakshyapati M, McGilchrist P, Ruhnke I (2019) Black soldier fly larvae does not compromise broiler meat quality. In '25th Biennial Conference Recent Advances in Animal Nutrition', 23-25 October 2019, Armidale, NSW, Australia. Abstracts of Short Presentations 2019, pg. iii. De Vilela JS, Alvarenga TIRC, Hopkins M, Kolakshyapati M, McGilchrist P, Ruhnke I (2020) Black soldier fly larvae in meat chicken diets modifies the fatty acid profile in chicken breast meat. In '31st Annual Australian Poultry Science Symposium', 16 – 19 February 2020, Sydney, NSW, Australia. Accepted.

Executive Summary

In seeking more sustainable and efficient food production, there is a global trend for alternative feed ingredients and especially protein source replacers (e.g. soybean meal) in livestock feed. In order to partially replace soybean and other ingredients in broiler diets, the maximum inclusion level of Black Soldier Fly (BSF) larvae were used in Ross 308 diets and fed to the broilers from 2 until 42 days of age. The 400 broilers were randomly assigned to one of the 5 dietary treatments, housed in 8 replicate cages with 10 birds each. The 5 dietary treatments included increasing levels of BSF larvae as follows: the starter diets (fed from day 2 -10) included 0, 2.5, 5, 7.5 and 10% BSF larvae, whereas, the grower (fed from day 11-21) and finisher diet (fed from day 22-42) included 0, 5, 10, 15 and 20% BSF larvae. Parameters of growth performance, nutrient digestibility, bird health and meat quality were evaluated. When fed during the whole experimental period (2 to 42 days), up to 20% of BSF larvae inclusion reduced significantly feed conversion ratio (FCR), increased body weight gain and final body weight. Ileal nutrient digestibility was only occasionally affected. A significant negative correlation of the BSF larvae could be observed on intraepithelial and blood lymphocytes, whereas increasing BSF larvae levels decreased these immune parameters linearly. The impact of the intestinal microbial population was minor where the abundance of *Enterococcus spp.* and an increase in *Dehalobacterium* was observed. Breast meat quality as well as the lean to fat ratio of the carcass remained unchanged except for the fatty acid profile of the breast meat: BSF larvae increased in the total amount of saturated fatty acids (SFA), eicosapentaenoic acid, and lauric acid, while it decreased the total polyunsaturated fatty acids (PUFAs) significantly. In conclusion, up to 20% of BSF larvae can be used in balanced broiler diets and fed from day 2 to day 42 without compromising the broiler's performance, nutrient digestibility, intestinal microbiota composition, and meat quality. Further research is required to investigate whether the effects of the BSF larvae on the immune system can have clinical impact e.g. health benefits.

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1. Introduction

The major protein source used in poultry and livestock feed in Australia is soymeal. However, due to non – ideal climate conditions and irrigation limitations, Australia has one of the smallest soybean productions in the world, ranking the least 30th position (M' Gee, 2011). The majority of the soy produced in Australia is used for human consumption, restricting the availability of soy for Australian poultry. Poultry products (meat and egg) consumption has steadily increased in Australia. The importations of soybean meal are currently 800 000 metric tonnes per annum and rely on the largest world soy producers such as Argentina (M' Gee, 2011).

The increasing world demand for soy, the constant fluctuant availability, and prices of soy make the Australian poultry industry vulnerable. Also, predictions of an increasing population along with a predicted increasing demand for chicken meat and egg. Resources such as water and land for agricultural production are becoming scarcer, which will further increase the competition and prices. Hence, alternative ways of supplying efficient and sustainable protein feed sources are warranted.

Several studies have investigated the feasibility of the Black Soldier Fly (BSF) larvae as a protein and fat source in poultry, swine, and aquaculture feed (Schiavone et al. 2017, Devic et al. 2018, Spranghers et al. 2018). Research shows that it is possible to achieve similar performance results replacing soymeal with BSF larvae meal in broiler and laying hen diets. We recently demonstrated that the replacement of 10% of the diet with BSF larvae for 6 weeks had no impact on hen performance and was associated with only minor changes on egg quality after being fed for 12 weeks (Ruhnke et al. 2018).

Morphological aspects of the BSF also are in favour of using this insect for feedstuff purposes. The nutrient contents of the BSF larvae can include up to 38% crude protein, including a balanced amino acid profile, up to 35% crude fat and up to 7% calcium (Ruhnke et al. 2018; De Marco et al. 2015). Besides the favourable nutritional potential of the BSF larvae, the potential benefit of antimicrobial compounds could also be identified. Lauric acid (12:0), is the main constituent of the BSF larvae fat, constitutes up to 64% of the total saturated fatty acids composition in the larvae. Lauric acid has been used as an antibiotic replacement in broiler diets, reducing bacterial count and improving intestinal health as well as broiler performance (Fortuoso et al., 2019; Londok and Rompis, 2019). Antimicrobial peptides and chitin are also constituents of the BSF larvae that have been previously demonstrated as efficient antimicrobial agents (Vogel et al., 2018; Elieh Ali Komi et al., 2018).

In Europe, the use of insect processed animal proteins for aquaculture animals was officially legalised in May of 2017 by the Commission Regulation (2017/883) (European Union 2017), while in Australia, insect use is regulated by the Stockfeed Standards and the Animal Feedstuffs Residue Standard.

Economically, as Australia imports most of its soy for animal production, soy is a limited and expensive source. Future independence from external markets and importations could increase the employment percentage and provide economic benefits to Australian industries and populations. However, it is important to highlight the fact that due to the currently high insect prices and a need to improve the techniques of the insect rearing practices, it may not provide immediate implementation in the animal feed industries.

2. Literature and intellectual property (IP) review

The use of the BSF larvae for human consumption and benefits are widely known in the research field and has been studied for over 30 years (Sheppard 1983, van Huis and Tomberlin 2017). The majority of research studies have focused on techniques for the larvae rearing process, development and quality of the BSF retained on different waste sources (Banks, Gibson et, al. 2014, Diener, Zurbrügg et, al. 2015, Tschirner and Simon 2015, Cheng, Chiu et al. 2017).

While the nutrient composition of BSF larvae is vital for feed formulation, the larvae quality including the dry matter, amino acid composition, ether extract quantity, fatty acid quality, and minerals can vary according to rearing process and rearing substrate (St-Hilaire et al. 2007, Nguyen et al. 2013, Spranghers et al. 2017). Crude protein values can varying from 339 to 431 g/kg dry matter (DM), ether extract from 218 to 386 g/kg DM, and crude ash from 197 to 27 g/kg DM. For example, when comparing four different substrates (chicken feed, biogas digestate, vegetable waste, and restaurant waste), the highest values of ether extract were found in larvae reared on restaurant waste, whereas, the highest values of ash content were found in larvae reared on biogas digestate (Spranghers et al. 2017).

Compared to the number of investigations performed on the larvae quality, growth, and development, only a few studies were done on the effect of the larvae on poultry diets (Devic et al. 2018, Dumas et al. 2018, and Wallace et al. 2018).

To date, a total of nine research studies have been published on BSF larvae in poultry feed focussing on either poultry performance, nutrient digestibility, or product quality. Seven of those studies were conducted in Europe, only three evaluated digestibility effects of larvae meal, only one used laying hens, and only one of the researches was performed in Australia/under Australian conditions (De Marco et al. 2015, Maurer et al. 2016, Marono et al. 2017, Schiavone et al. 2017a, Schiavone et al. 2017b, Altmann et al. 2018, Cutrignelli et al. 2018, Mwaniki et al. 2018, Ruhnke et al. 2018, Secci et al. 2018). One study focused on the health benefits of soy replacement with BSF larvae, including bird immunity and flock mortality (Lee et al. 2018).

The apparent nutrient digestibility, apparent ileal amino acid digestibility and apparent metabolizable energy of BSF meal have been investigated in broiler diets by exposing the flock to the experimental diets from day 26 to day 35 (De Marco et al. 2015). Significant differences of digestibility coefficients were found for full fat, partially defatted and highly defatted BSF meals (De Marco et al. 2105, Schiavone et al. 2017a). Despite these coefficient variations being available, animal trials evaluating the feasibility and impact of BFS meal on poultry performance do not take these digestibility coefficients into account, but base their feed formulation on total % replacement of soybean meal, not accounting for potential amino acid deficiencies (Cutrignelli at al. 2018).

Similarly, the inclusion levels of 5, 7, and 24% defatted and partially defatted BSF larvae meal were found acceptable on layer performance and egg quality when fed for the duration of 3 and 8 weeks (Maurer et al. 2016, Mwaniki et al. 2018). We previously evaluated the effect of 10% diet replacement with whole BSF larvae on flock performance and egg quality and found a minor impact for the duration of 6 and 12 weeks, respectively (Ruhnke et al. 2018). However, the short feeding time of BSF larvae in layer studies does not allow for conclusions on possible long-term effects as essential for commercial production.

Antimicrobial peptides of insects have been tested and proven successful against bacteria, including *Staphylococcus aureus* and *E. coli* (Chernysh et al. 2015; Elhag et al. 2017). This strategy of the insects may also be beneficial for the immune system and, subsequently, the health of animals that consume these insects (De Vilela et al. 2019). For instance, methanol extracts of BSF larvae and their effects on the growth, proliferation and viability of gram-negative bacteria have demonstrated that the larvae have strong antibacterial activities (Choi et al. 2012). Furthermore, full-fat BSF larvae (ether extract = 41.0%, lauric acid = 23.4%, DM basis) have been shown to reduce lactobacilli and D-streptococci in piglets and increased the survival rate of broiler chickens challenged with *Salmonella gallinarium* (Lee et al. 2018; Spranghers et al. 2018). Moreover, the chitin from insects are shown to stimulate immune response with no negative effects on broilers' growth performance, carcass and meat traits (Bovera et al. 2015 and 2016; Loponte et al. 2017). The presence of chitin, lauric acid and antimicrobial peptides in insects has been hypothesised to stimulate immune response with no negative effects (Bovera et al. 2015 and 2016; Loponte et al. 2017). In addition, chitosan, a molecule derivate from chitin, has been known to affect immune system of birds. Moreover, oligochitosan has been added to poultry diets as a prebiotic and enhanced immune functions (Huang 2007), affecting white blood cells and neutrophils of the host (Honda et al. 2016).

In conclusion, the determination of nutrient digestibility in broiler diets, maximum inclusion levels based on digestibility coefficients, and their long term impact on product quality (breast meat and carcass composition) have not been performed to date. The results obtained from this research will allow the commercial adoption of BSF larvae products for the poultry industry.

3. Objectives

The objective of the study was to investigate the impact of maximum inclusion levels of BSF larvae in broiler diets on bird's performance, nutrient digestibility, and immune cells from blood and the intestinal tract. The initial hypothesis were: (i) Increasing levels of BSF larvae in broiler diets would increase nutrient digestibility, (ii) not impact feed intake and increase feed conversion ratio, and (iii) improve immune parameters knowing that antimicrobial properties of the BSF larvae may act against pathogens.

4. Methodology

4.1 Experimental animals and diets

This research was approved by the Animal Ethics Committee at the University of New England (AEC18 - 084). After animal ethics approval, five experimental diets were formulated based on the breeder's manual (Ross 308, Nutritional specifications, 2016) for nutrient requirements of broilers. Diets for three development phases were formulated: starter diets (provided to broilers from day 2 to day 10 of their age), grower diets (provided to broilers from day 11 to day 21 of broiler's age) and finisher diets (provided from day 22 until day 42 of broilers age; Table 1). Samples of ingredients used were collected and chemically analysed in duplicate for gross energy, dry matter, crude protein, crude fat and ash determination. The results were introduced in the concept 5 program to formulate the experimental diets. Consistency on energy levels and digestible amino acid values were kept as close as possible in order to be able to compare broilers' performance between treatments. We replaced cottonseed oil with canola oil in the grower diets in order to provide sufficient linoleic acid supply in the diets. As this was done to meet the nutrient requirements, once can assume that the profile would not be affected in a matter relevant to the industry. Five inclusion levels of BSF larvae were investigated in the starter diets (0, 2.5, 5, 7.5 and 10%), and in the grower and finisher diets (0, 5, 10, 15 and 20%). In grower and finisher diets, the meat and soybean inclusion levels decreased with increased BSF larvae concentrations to adjust and balance for the nutrients provided by the BSF larvae. With both ingredients being of animal origin, one can assume that the impact on nutrient availability was minor.

4.1.1 Black Soldier Fly larvae and diet composition

The Australian company Karma3 reared and supplied the BSF larvae. All 4 BSF larvae batches used for this experiment were produced under the same conditions: fed the same conventional chicken feed, harvested on the 12th day of the larvae development and dried at 80°C for 8 days. Each batch of BSF larvae was chemically analysed to quantify values of gross energy, dry matter, crude ash, crude fat, crude protein and minerals (Table 2). Briefly, samples were freeze dried at – 50 °C and 5 Pa (FD – PILOT7 – 12 Series, Dynavac, Sydney, Australia). After being freeze dried, samples were ground in a Sunbeam Multigrinder II (Botany, NSW, Australia), with a wing blade system. Dry matter results were determined by freeze drying larvae samples and calculating the difference of fresh and dried samples. Crude ash was determined by incineration at 550 °C for 4 hours in a muffle furnace (Carbolite Gero Limited, Hope Valley, UK). Crude protein was estimated according to AOAC 1990, through nitrogen content estimation where Leco TrueMac Series Determinator based on a combustion technique (TruMac Series Determinator, LECO Corporation, St. Joseph, USA). The nitrogen content was multiplied per 6.25 to calculate the quantity of estimated of crude protein. Crude fat percentage of the larvae was measured through a Soxhlet extraction method (Folch et al. 1957). The amino acid profile of samples were analysed using the HPCL method (APAF SOP AAA-001), whereas the fatty acid composition was measured following the method described by (Clayton et al. 2012).

Table 1 The ingredients composition of the experimental diets (as - fed basis – calculated composition).

Item	(Starter Diets)[1]					(Growers Diets)[2]					(Finishers Diets)[3]				
	T1	T2	T3	T4	T5	T1	T2	T3	T4	T5	T1	T2	T3	T4	T5
Ingredient %															
Wheat grain	53.4	53.9	54.2	53.5	52.8	59.0	59.4	58.7	55.4	54.5	64.1	63.4	62.1	60.7	59.5
Soy	33.5	31.4	29.6	28.6	27.6	28.0	26.1	24.4	22.7	20.7	23.5	21.4	20.1	18.7	16.4
BSF larvae	0.00	2.50	5.00	7.50	10.0	0.00	5.00	10.0	15.0	20.0	0.00	5.00	10.0	15.0	20.0
Canola oil	3.16	2.37	1.63	1.02	0.41	4.29	2.88	1.83	1.52	0.72	0.00	0.00	0.00	0.00	0.00
Cottonseed oil	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.53	4.28	3.08	1.88	0.53
MBM	3.00	3.00	3.00	3.00	3.00	3.72	2.00	0.51	1.31	0.00	2.51	1.71	0.89	0.07	0.47
Hulled oat	3.00	3.00	3.00	3.00	3.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Celite	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Limestone	0.98	0.84	0.68	0.52	0.35	0.89	0.84	0.76	0.39	0.28	0.88	0.72	0.56	0.39	0.00
$CaHPO_4$	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phytase	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Salt	0.22	0.21	0.19	0.20	0.21	0.06	0.09	0.19	0.18	0.16	0.14	0.23	0.21	0.20	0.15
Na Bicarb	0.00	0.00	0.00	0.00	0.00	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
TiO_2	0.00	0.00	0.00	0.00	0.00	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vit. Premix[1]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Min. Premix[2]	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Choline	0.06	0.07	0.08	0.08	0.08	0.05	0.06	0.07	0.08	0.09	0.05	0.06	0.06	0.07	0.08
Lysine	0.19	0.19	0.18	0.15	0.12	0.66	0.30	0.26	0.19	0.16	0.18	0.14	0.07	0.01	0.00
Methionine	0.34	0.34	0.33	0.31	0.30	0.35	0.32	0.30	0.28	0.27	0.23	0.21	0.19	0.16	0.13

Isoleucine	0.00	0.00	0.00	0.00	0.00	0.08	0.06	0.04	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Arginine	0.00	0.00	0.00	0.00	0.00	0.11	0.13	0.15	0.14	0.17	0.00	0.00	0.00	0.00	0.00
Total	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Calculated composition															
ME, Kcal/Kg	3000	3000	3000	3000	3000	3100	3100	3118	3141	3167	3200	3200	3200	3200	3200
CP, %	23.8	24.0	24.2	25.0	24.6	22.7	22.7	23.0	24.2	24.5	19.9	20.4	21.1	21.8	22.8
d Met %	0.64	0.64	0.64	0.63	0.64	0.63	0.62	0.61	0.61	0.61	0.52	0.51	0.50	0.50	0.49
d Lys, %	1.28	1.28	1.28	1.28	1.28	1.53	1.28	1.28	1.28	1.28	1.16	1.17	1.17	1.18	1.23
d Arg %	1.42	1.39	1.37	1.37	1.37	1.37	1.37	1.37	1.37	1.37	1.12	1.10	1.10	1.10	1.10
d Ile %	0.86	0.86	0.86	0.88	0.89	0.86	0.86	0.86	0.86	0.87	0.71	0.72	0.71	0.79	0.81
Ca, %	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.79	0.79	0.79	0.79	0.80
P, %	0.45	0.46	0.47	0.50	0.48	0.45	0.45	0.45	0.45	0.45	0.40	0.40	0.40	0.40	0.44

[1] Starter diets were provided to the birds from day 2 to day 10.
[2] Grower diets were provided to the birds from day 11 to day 21.
[3] Finisher diets were provided to the birds from day 22 to day 42. [4]Vitamin concentrate supplied per kilogram of diet: retinol, 12000 IU; cholecalciferol, 5000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamin, 16 µg; biotin, 200 µg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg. [5]Trace mineral concentrate supplied per kilogram of diet: Cu (sulphate), 16 mg; Fe (sulphate), 40 mg; I (iodide),1.25 mg; Se (selenate), 0.3 mg; Mn (sulphate and oxide), 120 mg; Zn (sulphate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg.

Table 2
Black Soldier Fly larvae composition - Mean of 4 batches received from Karma3

				Amino acid profile (mg/g DM)				Fat Acid Profile (g/kg DM)			
Parameter	Mean	SEM	CV%	Amino acid	Mean	SEM	CV%	Fatty acid	Mean	SEM	CV%
Nutrients								Saturated fatty acids			
Dry matter (%)	94.2	0.251	0.01	Histidine	11.12	0.110	0.02	C12:0 (Lauric Acid)	128.3	6.542	0.10
Gross energy (MJ/kg)	22.6	0.161	0.01	Serine	16.13	0.243	0.03	C14:0 (Myristic Acid)	22.3	1.049	0.09
Crude protein	40.1	0.677	0.03	Arginine	18.42	0.393	0.04	C16:0 (Palmitic Acid)	32.7	1.268	0.08
Crude fat (%)	32.5	1.766	0.11	Glycine	19.94	0.309	0.03	C10:0	6.69	0.358	0.11
Crude ash (%)	14.0	0.217	0.03	Aspartic acid	32.74	0.398	0.02	C18:0	7.55	0.177	0.05
Minerals				Glutamic acid	43.68	1.056	0.05	Total SFA	200.7	9.209	9.18
Calcium (%)	4.82	0.147	0.06	Threonine	15.78	0.216	0.03	Monounsaturated fatty acids			
Phosphorus (%)	0.83	0.036	0.09	Alanine	27.62	1.318	0.10	C18:1n-9 (Oleic Acid)	24.65	1.004	0.08
Aluminium (%)	0.23	0.025	0.22	Proline	20.92	0.247	0.02	C18:1n-7	1.25	0.043	0.07
Copper (%)	0.02	0.000	0.03	Lysine	22.57	0.328	0.03	C18:1n-7t	0.51	0.105	0.41
Iron (%)	0.27	0.012	0.09	Tyrosine	21.01	0.304	0.03	C14:1n-5	0.32	0.016	0.10
Potassium (%)	1.33	0.048	0.07	Methionine	7.09	0.147	0.04	C17:1n-7	0.28	0.069	0.49
Magnesium (%)	0.39	0.009	0.05	Valine	23.16	0.279	0.02	Total MUFA	28.05	0.997	7.11
Manganese (%)	0.36	0.009	0.05	Isoleucine	17.23	0.251	0.03	Polyunsaturated fatty acids			
Sodium (%)	0.94	0.059	0.12	Leucine	27.27	0.378	0.03	C18:4n-3	0.32	0.040	0.25
Sulphur (%)	0.34	0.006	0.04	Phenylalanine	16.97	0.264	0.03	C20:5n-3	0.11	0.012	0.22
Zinc (%)	0.14	0.001	0.01	Total	341.66	4.088	0.02	C18:2n-6 (Linoleic Acid)	20.2	0.833	0.08
								C18:3n-3	2.35	0.106	0.09
								Total PUFA	23.46	0.859	7.32

4.2 Growth performance

Parameters of broiler performance include feed conversion ratio (FCR), individual feed intake, individual weight gain, body weight and mortality. Mortality was daily recorded, whereas, the total weight of the birds per cage was recorded 4 times during the experimental period (day 2, day 10, day 21 and day 42) and feed weight was recorded every time any feed was provided.

Feed intake of the broilers was measured by recording the summary of ''feed in'' (all the feed weight that was placed in the cage feeder) and the ''feed out'' (the weight of the remaining feed in the feeder of each cage at the end of each phase). At the end of each phase: starter (2d to 10d), grower (11d to 21d) and finisher (22d to 42d) feeder of each cage was individually weighted and the value recorded. The total feed intake of the phase per cage was calculated as follows:

$$\textbf{Cage feed intake} = \text{Sum (cage }''\text{feed in} - \text{ cage }''\text{feedout}'')$$

After calculating the ''Cage feed intake" of each cage, the FCR was then calculated by the feed intake of the animals divided by the body weight gain of each cage. The body weight gain was corrected using the recorded mortality weight of each cage within each phase (starter, grower and finisher phases).

The mortality correction was done as described below:

$$\textbf{FCR} = \frac{\text{Cage feed intake}}{((\text{Final weight of cage} + \text{ weight of dead birds}) - \text{Initial body weight of cage}))}$$

The individual feed intake was calculated from FCR and individual weight gain values, such as follows:

Individual feed intake = FCR*Individual weight gain

Individual body weight gain was calculated by subtracting values of the individual body weight of birds per cage at the beginning of the phase (starter, grower and finisher) to the individual body weight of broilers at the end of the same phase. Whilst the individual body weight of the broilers at the start of each phase was calculated by dividing the total body weight per number of broilers at day one of each phase and individual body weight at the end of the phase calculated by dividing the total body weight of the cage at the end of the phase by the number of birds at the end of the phase.

4.2 Nutrient digestibility

4.2.1 Sample preparation

At the 21 days of age, 5 birds per cage (total of 200 birds) were sampled, and the content from the distal 2/3rd of the ileum was collected into a container (pooled from 5 birds) and immediately placed on ice before being stored at – 20˚C until further analysis. On the day 42 of the broiler's age, 3 broiler's per cage (total of 120 birds) were sampled and the ileal digesta content were collected (pooled from 3 broilers) and stored at -20˚C. All samples were freeze-dried (FD – PILOT7 – 12 Series, Dynavac, Sydney, Australia) for 7 days. The condenser temperature and the vacuum pressure was maintained at – 50 ℃ and 5 Pa. After freeze drying, samples were grounded in a Sunbeam Multigrinder II (Sunbeam, Botany, NSW, Australia), with a wing blade system. Homogenised samples of grower and finisher diets were also grounded and prepared for nutrient content analysis.

4.2.2 Apparent ileal digestibility

For titanium dioxide (TiO_2) analysis, approximately 0.1 g of dried and grounded ileum content and ~ 0.2 g grounded feed was accurately weighted, recorded and ashed in a muffle furnace (Carbolite Gero Limited, Hope Valley, UK) at 580℃ for 13 hours. Then, the spectrophotometry method was followed as described by Short et al., (1996). The equation to calculate the TiO_2 percentage in the samples was:

TiO_2% = Abs (sap)*50/slope/W (sap) g/10

In order to calculate the slope, the following equation was used:

Slope = Abs/Con

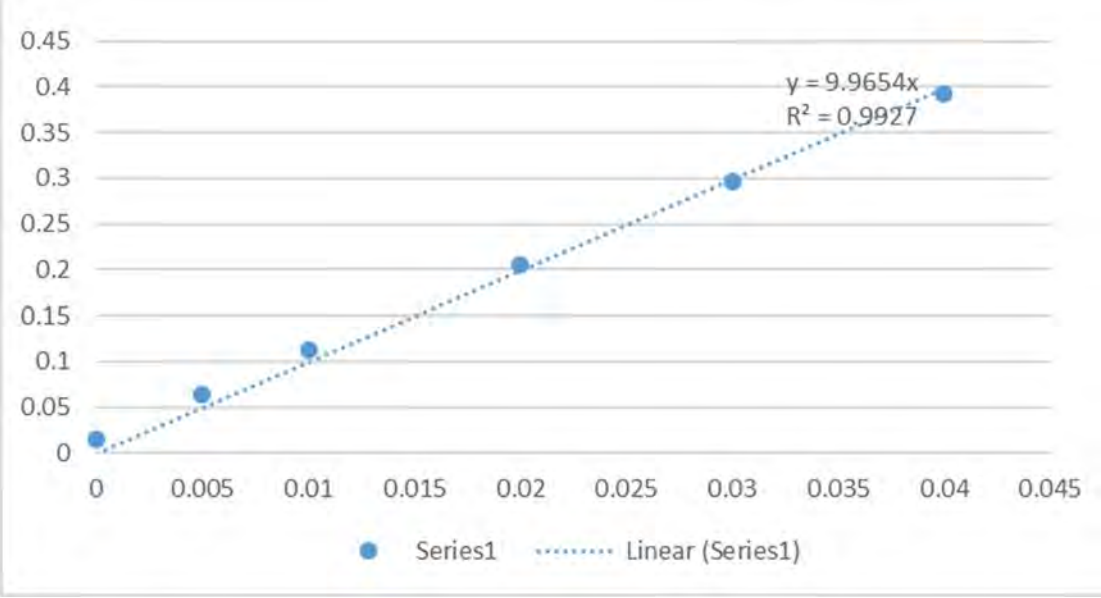


Figure 1: An example of the slope calculation in excel to determine the TiO_2 content of the samples.

After quantifying the titanium dioxide content in the experimental feed and ileum content, the apparent ileal digestibility of nutrients was calculated according to the following equation:

$$\text{Apparent ileal digestibility} = 100 - [(\text{TiO2 feed \%/ TiO2 ileum\%}) * (\text{Nutrient ileum \%/ Nutrient Feed\% })] * 100$$

4.2.3 Crude protein determination

Nitrogen content was determined by a LECO FP – 2000 automatic nitrogen analyser (Leco Corporation, St. Joseph, MI, USA) and the method used was combustion method (Nelson and Sommers 1982). Nitrogen content was multiplied by 6.25 to calculate estimated crude protein values.

4.2.4 Crude fat determination

Crude fat was determined by Soxhlet extraction method (Folch et al. 1957). Briefly, thimbles were labelled and placed for 4 hours in a furnace oven at 105 ℃. Then, approximately 2 g of grounded samples were accurately weighted inside of each thimble and dried at 105 ℃ overnight. Dry matter of samples was then measured and placed in the Soxhlet apparatus for fat extraction. On the upcoming day, samples were removed from the Soxhlet apparatus, dried at 105 ℃ overnight and finally the weight of the defatted samples were carefully determined.

The calculations used to determine fat % were:

Dry sample weight (g) = dried (thimble + cotton + sample) weight – dried (thimble + cotton) weight.

Dry matter % = dry sample weight/''as is'' sample weight*100

Fat weight (g) = dried (thimble + cotton + sample) weight – dried (thimble + cotton + extracted sample) weight

Fat % = fat weight (g)/dry sample weight (g) *100

4.2.5 Dry matter determination

Crucibles were labelled and placed into a muffle furnace (Carbolite Gero Limited, Hope Valley, UK) at 105℃ overnight (for 16 to 24 hours). In sequence, crucibles were taken out of the 105℃ oven and cooled for 40 to 50 minutes in a desiccator before being weighted. After recording dried crucible weight, 1 to 3 g of samples (previously freeze dried and grounded ileum content) were weighted in the previously weighted crucibles and kept in the 105℃ oven overnight for dry matter results. After collecting dry matter results, the samples were kept in muffle furnace (set at 350℃ for 1 hour, then at 600℃ for 2.5 hours, and then to 105℃ for 1-2 hours) to obtain ash. After processing, cooled ashed samples were placed in a desiccator for 40-50 minutes and then weighted to determine the amount of crude ash.

The calculations used for ash values were:

$$\textbf{Dry matter } \% = \frac{\text{dried sample weight (g)}}{\text{wet sample weight (g)}} * 100$$

$$\textbf{Ash } \% = \frac{\text{ashed sample weight (g)}}{\text{dried sample weight (g)}} * 100$$

4.2.6 Mineral determination

Ashed samples were then saved and used to quantify minerals: Ca, P, Al, Cu, Fe, K, Mg, Mn, Na, S and Zn using Ultrawave Microwave Digestion technique for nitric acid digestion, a variation method on application Note UW21 SRM sample NIST 1575. This method promotes an acid digestion of samples in a Single Reactor Chamber using temperature and microwave heating for the metal determination by spectroscopic methods.

4.3 Bird health

At 21 and 42 days of age, blood from two broilers per cage were collected for analysis of blood cells and intestinal microbiota analysis. At 42 days of age, intraepithelial lymphocytes were determined.

4.3.1 Total blood count

To determine blood cells and immune parameters, samples from same broilers (2 broilers/cage) were collected at two different ages (day 21 and day 42 of age). At day 21, whole blood from the brachial vein (wing vein) was collected from 2 broilers per cage and placed in 4 mL plastic K_2 EDTA tubes (BD Vacutainer, Plymouth, UK). All broilers who had their blood collected were leg banded to be identified on day 42. On day 42, the appropriate 2 broilers per cage were identified through their leg bands and humanely sacrificed for blood collection as well as to determine the intestinal intraepithelial lymphocyte (IEL) population.

Total blood count was analysed within 2 hours after collected, using a CELL-DYN 3700 analyser (Abbott laboratories, USA), applying a validated method (Abbott et al. 2003). Hematology parameters measured included total leucocytes (WBC), neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS), basophils (BASO), total erythrocytes (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelets (PLT). Means for each replicate within each time point were calculated and used for statistical analysis.

4.3.2 Flow cytometric analysis (FACS) analysis

In order to compare different immune system responses in the broiler body, epithelial lymphocytes (Cytotoxic T – cells CD4 – CD8+, T – helper cells: CD4+ CD8-; Double Positive Cells CD4+ CD8+) were measured. Intraepithelial lymphocytes were isolated from the small intestine of 40 broilers (1 bird per cage) when birds were 42 days of age following the method used by (Röhe 2014). The intestinal cell preparation was stained with either a cocktail of T lymphocyte CD marker antibodies (CD3-AF647, CD4-FITC and CD8-PE – purchased from Southern Biotech - Birmingham, AL, USA) or a cocktail of isotype control antibodies according to the manufacturer's recommendations for 30 minutes on ice and in the dark. Flow cytometric data was acquired on an Amnis FlowSight imaging flow cytometer (Amnis, Seattle, WA, USA) CD4-FITC and CD8-PE were excited using the 488 nm laser and the CD3-AF647 was excited using the 633 nm laser. The flow cytometric data were analysed using IDEAS software version 6.0.340.0 (Tree Star, Inc.,Ashland, OR, USA). Signals from the isotype antibody cocktail was substracted from the T lymphocyte CD antibody fluorescence. From the total cell population that was acquired, the CD3+ intact cell population was gated. Sub-gates were applied to the intact CD3+ cells population to determine the proportion cytotoxic T lymphocytes (CD3+CD8+), T helper lymphocyte (CD3+CD4+) and CD4+CD8+ double

stained lymphocytes (CD3+CD4+CD8+) in the total intestinal cell population isolated from the intestines of broilers fed up to 20% BSF larvae in their diets.

4.2.3 DNA extraction and sequencing

The caecal DNA was extracted using DNeasy PowerSoil Pro kit (Qiagen, Inc., Doncaster, VIC, Australia) with some modifications. In brief, approximately 300 mg of glass beads (0.1mm) and 60 mg of frozen caecal contents were placed in a 1.5 mL colourless Eppendorf tubes 3810X (Eppendorf AG, Hamburg, Germany). Then 800 µl of solution CD1 (Qiagen, Inc., Hilden, Germany) was pipetted to samples prior the disruption of the cells by Tissuelyser II (Qiagen, Inc., Hilden, Germany) for 5 min at frequency 30/seconds. The samples were incubated on a heating block at 90 ºC for 10 minutes and then centrifuged (centrifuge model 5424 R, (Eppendorf AG, Hamburg, Germany) at 20,000 × g for the duration of 5 minutes. The supernatant was transferred onto a loading block and extraction was performed using QIAcube HT robotic (Qiagen, Hilden, Germany). A QIAcube HT plasticware kit was used and the reaction buffers (CD3, AW1, AW2, 96% Ethanol or CD6 – Qiagen, Hilden, Germany) were loaded onto a dedicated cassette. In addition, the filtration and elution blocks were placed inside the machine and the extraction was performed following the manufacture's protocol (QIAamp 96 DNA QIAcube HT Q Protocol).

4.4. Meat quality

At 42 days of age, breast meat from two broilers per cage were sampled for analysis of pH, colour, cooking loss, shear force, lipid oxidation, amino acid profile, fatty acid profile, meat yield and CT scan images for carcass composition.

4.4.1. Lipid oxidation

Immediately after obtaining the broiler breast meat, consistent pieces from the same portion of the left chicken breasts were cut and sealed in vacuum bags and in order to prevent samples to oxidate. All 80 samples (2 broilers per cage, 16 broilers per treatment) were stored at – 80˚C until analysis. Thiobarbituric acid – reactive substances (TBARS) was used to determine the lipid oxidation of the broiler breast meat.

4.4.2. Thiobarbituric acid reactive substances (TBARS) Methodology description:

For lipid oxidation, 100 mg of frozen samples were combined with 500.0 µL radio-immunoprecipitation assay (RIPA) buffer (Item No. 10010263, Cayman Chemical Company Ltd., Missouri, USA) and homogenised using micro-tube pestles (Zhang et al 2019). The supernatant was isolated and TBARS contents were determined as per the TBARS (TCA Method) Assay Kit colorimetric protocol (Item No. 700870, Cayman Chemical Company Ltd., Missouri, USA). This used a benchtop spectrometer (model FLUOstar OPTIMA™, BMG Labtechnologies, Victoria, AUS) set to measure absorbance at 540 nm. Technical duplicates were averaged and data were expressed as mg malondialdehyde (MDA) per kg of (wet) sample.

4.4.3. Colour and pH

At 42 days of age, two broilers per cage were sampled for analysis of pH, colour, cooking loss, shear force, lipid oxidation, amino acid profile, fat acid profile and CT scan images for carcass composition. After obtaining the breast meat, the samples were placed in a sealed plastic bag and chilled overnight at 4 ℃. On the following day, three colour measurements per sample were taken parallel to the fibre position on the bloomed surface (blooming for 35 minutes) using a Minolta Chroma Meter CR 300 (Minolta Co., Ltd., Japan), and then averaged. Measurements were recorded in L* a* b* (L* = lightness, a* = redness, b* yellowness) colour space - defined by the Commission Internationale de l'Eclairage in 1976 (Anon. 1998). Measurements of pH were done by a direct insertion of spear – head pH probe (IJ44C probe, Ionode, Pty Ltd., Australia) using a digital pH probe (IJ44C probe, Ionode Pty Ltd., Tennyson, Queensland, Australia) with a combination electrode (glass body with a spear tip). One temperature and 2 pH readings were taken for each sample, with the latter averaged.

4.4.4. Cooking loss and shear force

Cooking loss and shear force analysis was performed following the Lyon and Lyon (1996). Chicken breasts of 120 broilers were individually stored in sealed plastic bags and chilled at 4 ℃ overnight. Uniform pieces of approximately 65g were then cut, weighed (fresh sample weight) and placed in vacuum packed bags, which were stored at -20˚C until the analysis. A water bath (Model: BTC 9090) was heated at 85℃ and samples were placed into the water bath for 25 minutes +/- 1 minute. The sample batches were then cooled in a sink filled with cold running tap water for 30 min +/- 1 minute and then removed from the bag and weighted (cooked sample weight) for cooking loss determination.

$$\textbf{Cooking Loss}\ (\%) = \frac{\text{Cooking loss (g)} * 100}{\text{Fresh sample weight (g)}}$$

For shear force analysis, the cooked, cooled and weighted breast meat samples were placed at 4 ℃ overnight. On the following day, the cooked samples were cut into subsamples for textural analysis. Details of sample thickness, shape and fibre orientation for samples used for shear force and compression measurements are given in Bouton et al. (1971) and Bouton and Harris (1972). All textural measurements were made on a Lloyd Instruments LRX Materials Testing Machine fitted with a 500N load cell (Lloyd Instruments Ltd, Hampshire UK). Briefly, 6 subsamples with a rectangular cross section of 15 mm wide by 6.66 mm deep (1 cm^2) were cut from each block, with fibre orientation parallel to the long axis, and at right angles to the shearing surface. The force required to shear through the clamped subsample with a triangulated 0.64 mm thick blade pulled upward at a speed of 100 mm/min at right angles to fibre direction was measured as kg peak force (greatest force measured on the force-deformation curve). Values of the kg peak force were recorded and the mean of 5 to 7 subsamples was calculated. Two wedges were cut from the remaining sample so that the muscle fibre direction was parallel to the largest cut surface, and the thickness at the centre of the wedge was about 10 mm. The wedge-shaped sample was placed on the testing plate so that the muscle fibres were parallel to the plate and the surface of the sample just touched the bottom of a blunt cylindrical metal rod (diameter 6.3 mm) that was positioned 10 mm above the plate. This rod was then driven 8 mm into the sample at a speed of 50 mm/min, twice at exactly the same position. The wedge was then moved and another reading taken. From 5 to 7 measurements were made per sample, with mean compression recorded in kilograms. Compression was calculated as the product of hardness and cohesiveness, where hardness is the maximum height of the first force-deformation curve, and cohesiveness is the work done

by the second compression stroke (area under the second force–deformation curve), divided by the work done by the first compression stroke (area under the first force–deformation curve).

4.4.5 Carcass composition (lean and fat ratio)

Computed Tomography (CT) scanner (HiSpeed Qx/I, Milwaukee, WI, USA) was used to collect images from 120 (3 broilers per cage/ 24 broilers per treatment) broilers (42 days old) carcasses. An average of 32 scans were taken per animal. The radiation tube operated at 120 (tube voltage) KV and 100 mAs (radiation intensity), using a 5 mm thickness, 10 mm spacing, a pitch of 1.5 and field of view of 500. The direction of the scans was cranium-caudal. Images were processed using an open source image processing package called ImageJ bundled with 64-bit Java 1.8.0_112 downloaded at: https://imagej.net/Downloads. Slices of images were separated into files, each file corresponded initially to 2 birds. On imageJ, images were cut separating birds from the bed and keeping only one bird per file. Single files for each bird, containing all slices of each bird were analysed for total fat and total lean (muscles) in grams (Figure 2, 3). After the image sections were cut, a script was used on ImageJ bundled with 64-bit Java 1.8.0_112 to identify through the images and calculate the quantity (in grams) of the following parameters: primal weight, lean muscle mass, muscle mass, total fat content, total protein content, total bone content, and total ash content.

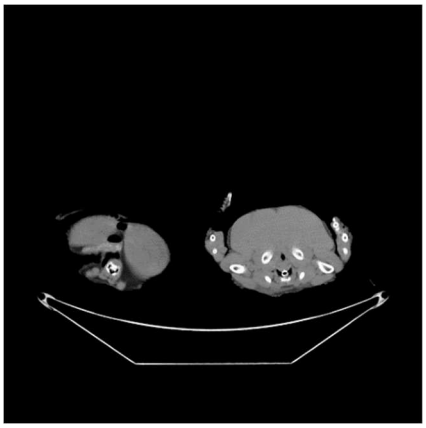


Figure 2 - An example image of the CT evaluation of broiler carcass composition. Craniocaudal view of two broilers where the section cut was 5 mm and the mAs and kV settings 150 and 120, respectively.

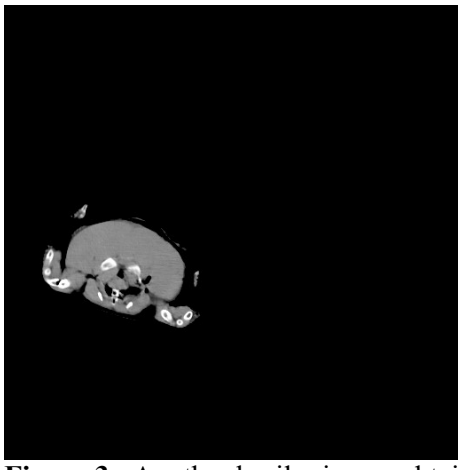


Figure 3 - Another broiler image obtained during CT evaluation. Grey indicates soft issue, e.g. muscle tissue, whereas the while structures represent bone tissue. With the carcass laying on its dorsum, the voluminous breast meat (grey) becomes very prominent.

4.5 Amino acid profile

Consistent pieces of approximately 45 g breast meat were collected from the same anatomic location of the right broiler breasts, cut into pieces, placed into 50 ml tubes and stored at – 20˚C until being freeze dried. Samples were placed in the freezer dryer machine (FD – PILOT7 – 12 Series, Dynavac, Sydney, Australia) at -55 ℃ and 6 Pa for the duration of 7 days. All 80 samples (2 birds per cage, 16 birds per treatment) were ground. HPCL analysis were performed as per APAF SOP AAA-001 at Macquarie University. Briefly, samples underwent 24 hours liquid hydrolysis in 6M hydrochloric acid at 110 °C. Under these conditions, asparagine was hydrolysed to aspartic acid and glutamine to glutamic acid; therefore, the reported amount of these acids is the sum of those respective components. After hydrolysis, all amino acids were labelled using the Waters AccQTag Ultra chemistry (Totowa, NJ, USA), following supplier's recommendations, and analysed on a Waters Acquity (Waters Corporation, Milford, MA, USA) Ultra Performance Liquid Chromatography (UPLC).

4.6 Fatty acid profile

Consistent pieces of approximately 45 g breast meat were collected from the same anatomic location of the right chicken breasts, cut into pieces, placed into 50 ml tubes and stored at – 20 ˚C until being freeze dried. Samples were placed in a 12 L freezer dryer machine (FD – PILOT7 – 12 Series, Dynavac, Sydney, Australia) for 7 days. The condenser temperature and the vacuum pressure was set at – 50 ℃ and 5 Pa. After freeze drying, samples were grounded in a Sunbeam Multigrinder II (Botany, NSW, Australia), with a wing blade system. Two breast meat samples obtained from 2 different broilers per cage, 16 samples per treatment were analysed using the method described by Clayton et al (2012).

5. Statistical analysis

All statistical analysis were performed in SPSS (v22, IBM, Amork, NY, USA). Data of performance, nutrient digestibility and total blood count were analysed for best curve fitting representation according to the larvae inclusion level responses for the different parameters and the P value and R square for the best curve fitting was represented on the result figures. Total blood count results were log transformed before statistical analysis. To analyse flow cytometry results, it was performed univariate linear regression. Data of meat quality (pH, colour, cooking loss, shear force, amino acid, fatty acid profile, and carcass composition) were analysed using One-way ANOVA. For the analysis of microbiota sequenced data, Qiime v.1.3.0 software was used.

6. Results and Discussion

6.1 Growth performance

A negative quadratic effect (P = 0.043, R2 = 0.09) for feed conversion ratio was observed during the starter period as levels of BSF larvae were increasingly included in the diets (Figure 5). On contrary, during the grower period (from day 11 to 21 of age), there were no significant differences in performance parameters when increasing levels of larvae were included in the diets (Figure 6), except for individual body weight, where there was a linear positive response (Figure 6d). However, a best curve fit for negative cubic responses (P = 0.034, R^2 = 0.16) representing a negative decrease in the feed conversion ratio and a positive linear response was observed during the final period (22 to 42-day old birds) indicating increase in body weight of the birds with increasing levels of larvae in the diets. During the total experimental period, FCR (Figure 4a) of the broilers was reduced as the larvae inclusion levels increased in the diet (P = 0.004) which is in agreement with the results obtained from Bovera et al. (2016) who also found low FCR of broiler chickens fed with insect meal (5% to 15% of inclusion) than the control diet using soybean meal. Therefore, addition of BSF larvae seem to be beneficial even though the R value was very low. Further research with significant more data points would be warranted to allow for more reliable conclusions and investigate the correlation strength further.

During the starter period (from day 2 to 10 of age), there were no significant differences in feed intake, body weight gain and body weight at day 10 (Figure 7). During the grower period, final body weight (Figure 6d) showed a positive quadratic increase in body weight when larvae levels were increasingly added to the bird's diets (P = 0.01, R^2 = 0.22). However, again during the final period (22 to 42-day old birds), there was no significant differences in feed intake or body weight gain (Figure 7). When body weight gain and individual body weights were calculated for the total experimental period, there was a significant positive linear regression (P = 0.027). Similarly, Khan et al. (2018) has also observed a positive impact of insect inclusions achieving higher live weight in broiler chicks fed with insect larvae (silkworm inclusion level 7%, housefly maggot inclusion level 8%, *Tenebrio molitor* inclusion level 8.1%) compared to broilers of the control group. However, total feed intake in this study was not significantly affected by the larva inclusion in the bird's diet which suggest that the experimental diet might be more efficient than control diet. In contrast, Onsongo et al. (2018) did not find any effect of insect diets on performance parameters (feed intake, FCR, bodyweight gain) in broiler chickens when being fed BSF at different inclusion levels (13.8%, 27.4% and 42.0% of crude protein in starter feed and 11.0%, 37.2% and 55.5% of the crude protein in finisher diet). We therefore conclude that BSF larvae can be fed up to 20% in a balanced diet without any negative effects on final live weight, FCR, body weight gain, and feed intake. However, it is pivotal to highlight that the diets were formulated aiming to meet the required digestible amino acids and there were numerous details in the feed formulation that could have affected performance of the birds. We therefore conclude that full-fat BSF larvae in broiler diets can be added up to 20% in broiler diets without compromising performance parameters. However, it is not possible to entirely affirm that the improvements in broiler performance are entirely related to the BSF as an ingredient itself, but may influenced by a combination of factors in the diet formulation. In order to fit the BSF larvae in industry

standards for diet formulation, it is recommended to use of lower levels of full-fat BSF larvae or the include defatted BSF larvae.

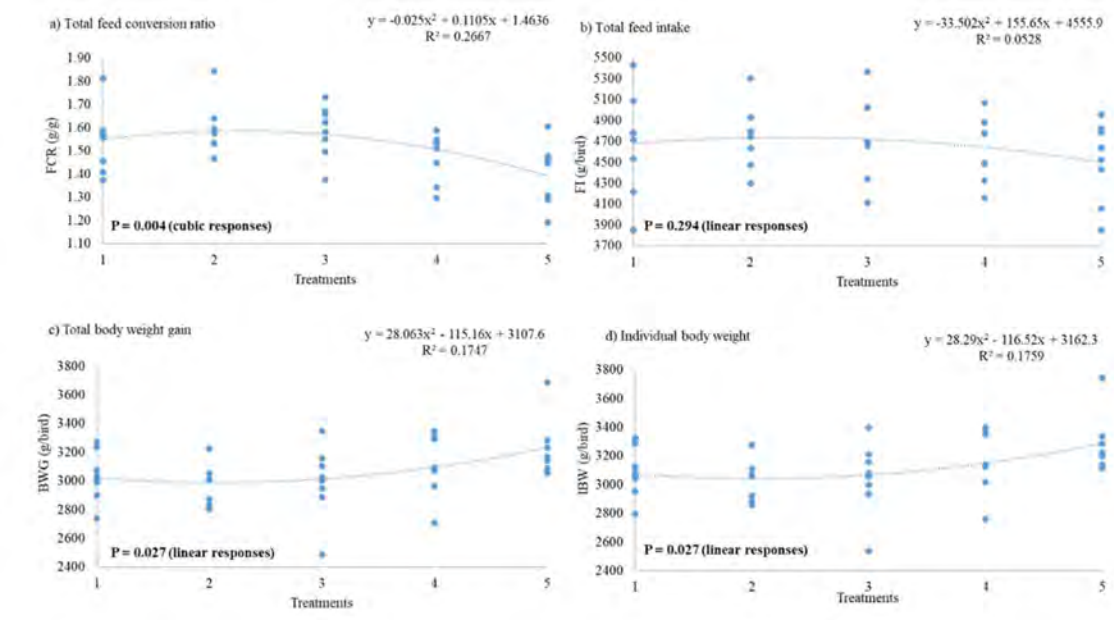


Figure 4 – Growth performance of broilers (Ross 308) from day 2 to 42 of age with different levels (0, 5, 10, 15 and 20%) of BSF larvae inclusion in the diet.

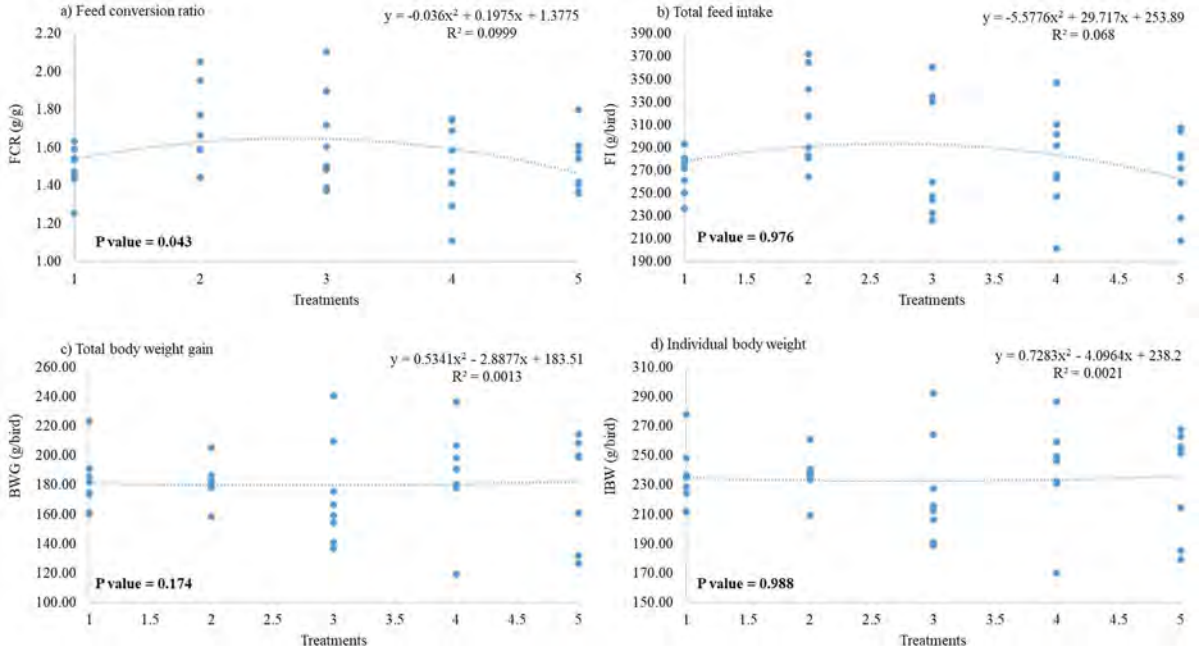


Figure 5 – Growth performance of broilers (Ross 308) from day 2 to day 10 of age with different levels (0, 5, 10, 15 and 20%) of BSF larvae inclusion in the diet.

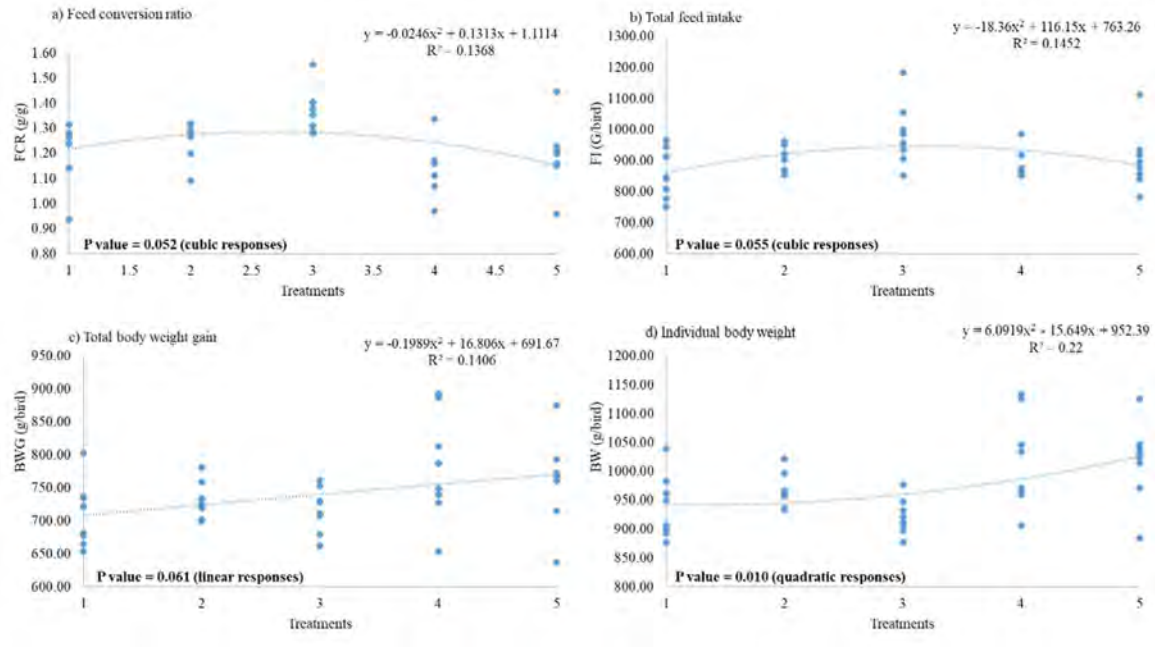


Figure 6 - Growth performance of broilers (Ross 308) from day 11 to 21 of age with different levels (0, 5, 10, 15 and 20%) of BSF larvae inclusion in the diet.

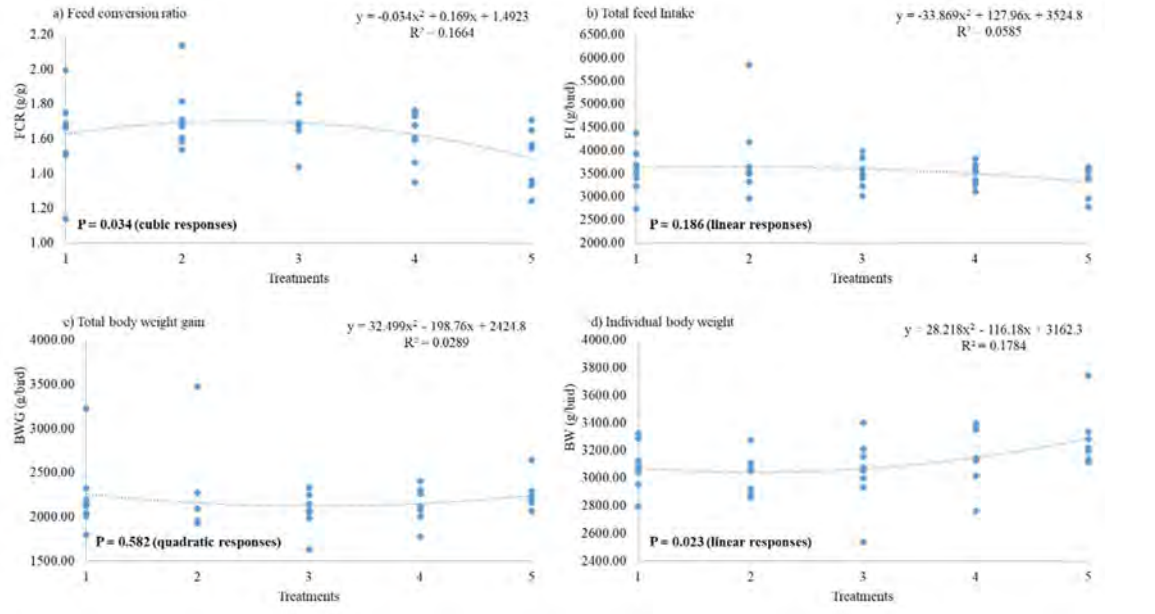


Figure 7 - Growth performance of broilers (Ross 308) from day 22 to 42 of age with different levels (0, 5, 10, 15 and 20%) of BSF larvae inclusion in the diet.

6.1 Nutrient digestibility

There was no effect on dry matter, crude protein or ash digestibility in this study even after addition of 20% of larvae in broiler diets at day 21 (Figure 8). No effect was observed on crude ash or crude protein digestibility at day 42, but a negative quadric relationship was observed in the dry matter digestibility (P=0.037; Figure 9). Bovera et al. (2016) also showed decreased apparent ileal DM digestibility in broiler chicken by addition of insect meal with a complete replacement of soybean meal by *Tenebrio molitor* larvae meal.

No difference in crude protein digestibility neither at day 21 or day 42 of the broilers was observed in this study possibly because of the adaptation to the chitin levels in the diet, which was also reported in ducks fed with insect larvae in a study by Gariglio et al. (2019). Bovera et al. (2016) reported a reduction in dry matter digestibility and crude protein digestibility (approximately 2 and 7% of decrease) when 100% soybean meal was replaced with *Tenebrio molitor* larvae in 62 days old shaver brown broiler diets. Cutrignelli et al. (2018) reported a reduction in dry and organic matter digestibility as well as crude protein digestibility when soybean meal was replaced by BSF larvae by 100% in 24 weeks Lohman Brown Classic laying hen diets, while the lipid digestibility remained unaffected. However, the inclusion of 17% BSF larvae meal as a total replacement of the soybean meal in laying hen (24-45 weeks of age) diets reduced feed intake, live weight and crude protein digestibility, whereas, lipid digestion was not affected (Di Meo et al. 2017; Cutrignelli et al. 2018). In this study, the quadratic component of the variance was significant (P=0.04) for the fat digestibility for the treatment groups at day 21 with increasing levels of BSF larvae. However, increasing the levels of BSF larvae did not affect fat digestibility at day 42 of the broilers (Figure 9c). On the other hand, Cutrignelli et al. (2018) and Bovera et al. (2018) found that the apparent ileal ether extract digestibility in laying hens was similar for BSF meal- (25 and 50% inclusion level) when comparing to hens fed soybean meal.

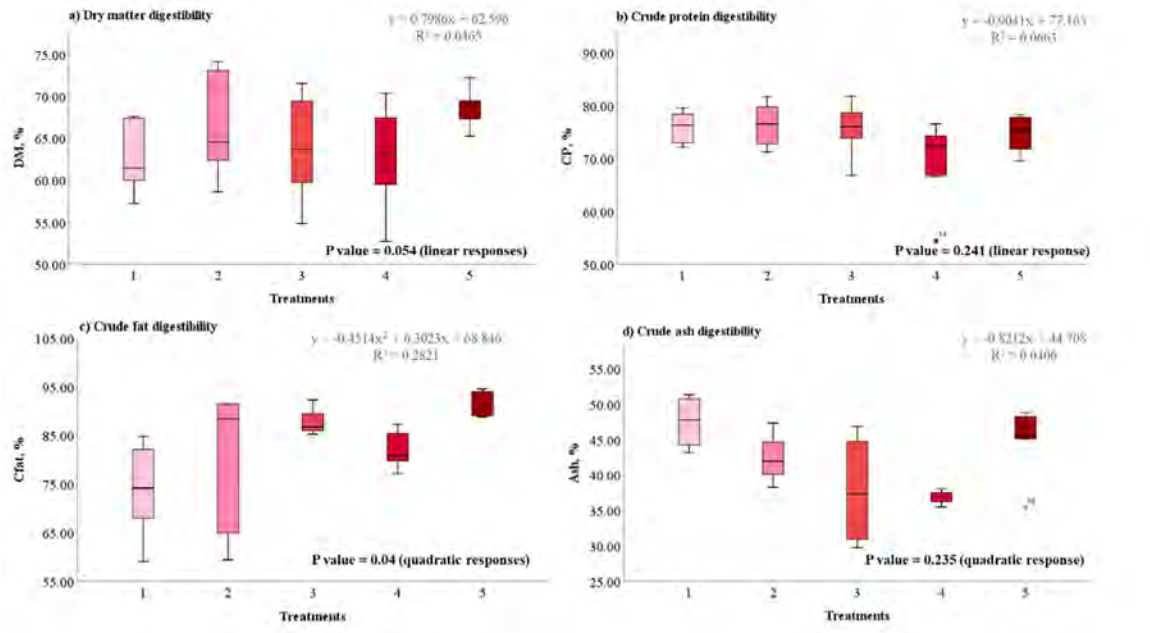


Figure 8 - Digestibility results from day 21 of broilers fed with different levels (0, 5, 10, 15 and 20%) of BSF larvae in the diet.

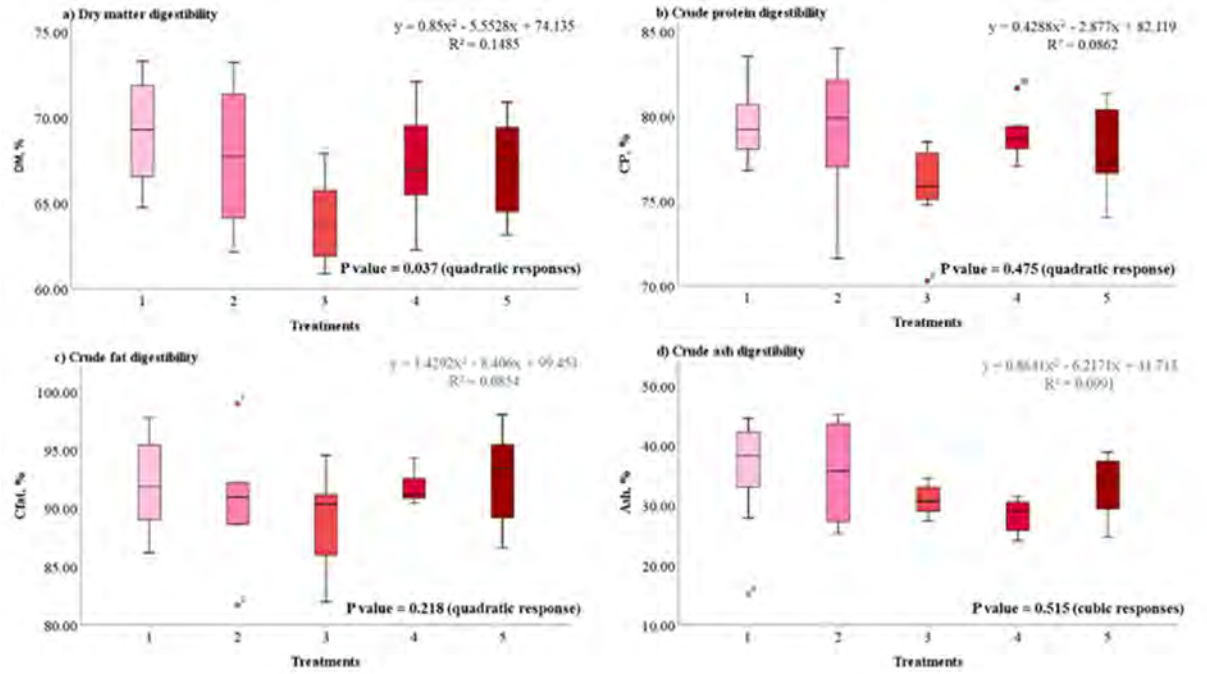


Figure 9 - Digestibility results from day 42 of broilers fed with different levels (0, 5, 10, 15 and 20%) of BSF larvae in the diet.

6.2 Immune response

6.3.1 Total blood count results

The inclusion of BSF larvae in the broiler diets reduced white blood cells from 50.2 (10^6 /ml) in the blood to 32.2 (10^6 /ml) when measured at day 21. The lymphocytes in the blood cells also reduced by 50% (from 39.4x10^6/ml to 20.7x10^6/ml of blood) at day 21 of bird's age. There was also a significant difference in the neutrophil count, where T2 (5% of larvae inclusion) had the lowest value compared to T4 (15% of larvae inclusion) which had the highest value. Interestingly, a study by Schiavone et al. (2017) showed that the replacement of up to 100% of soybean oil in broiler diets by the BSF larvae oil did not affect blood parameters or health status of the birds which may indicate that high levels of lauric acid may not be the reason why white blood cells and lymphocytes reduced in the bird's immune cells.

A negative quadratic response in white blood cells (P = 0.001, R^2 = 0.40) and lymphocytes (P = 0.001, R^2 = 0.40) were observed (Figure 10) representing a decrease in both cells in the blood as larvae levels were increased in the diets at day 21 of the broilers. Similarly, for broilers at 42, best curve fitting for a negative linear response (P = 0.027, R^2 = 0.06; Figure 11) was observed again representing a decrease in white blood cells when inclusion rate of larvae increased in the diets. A positive cubic response (P = 0.006, R^2 = 0.09) was observed for neutrophils as the BSF larvae was increased in the diets. There was no significant response to the mean corpuscular volume (Figure 10d) as well as to the other blood cells analysed (monocytes, eosinophils, basophils, red blood cells, haemoglobin, haematocrit, mean corpuscular haemoglobin concentration and platelet) at this period. However, mean corpuscular volume was found to (Figure 11d) linearly increase (P = 0. 002, R^2 = 0.12) at day 42 while other blood parameters remained unaffected.

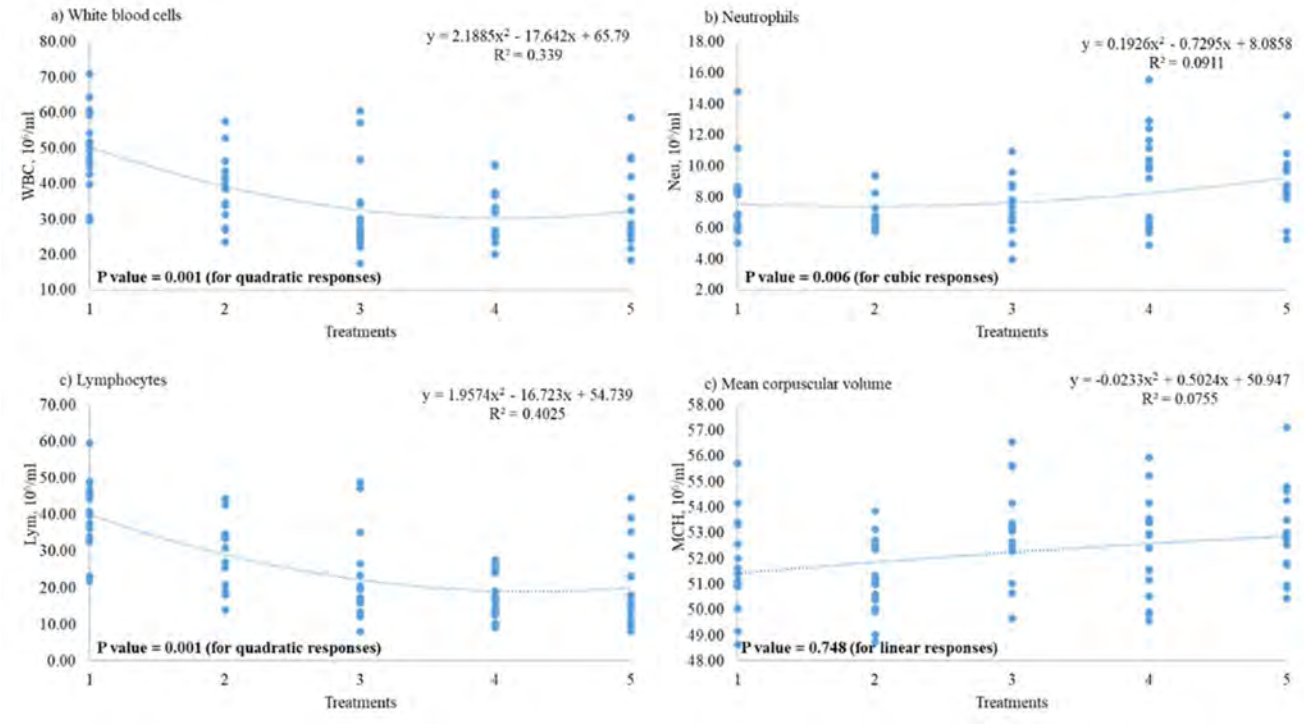


Figure 10 –Total blood count of 21days old broilers fed with BSF larvae with different inclusion levels (5%, 10%, 15% and 20%).

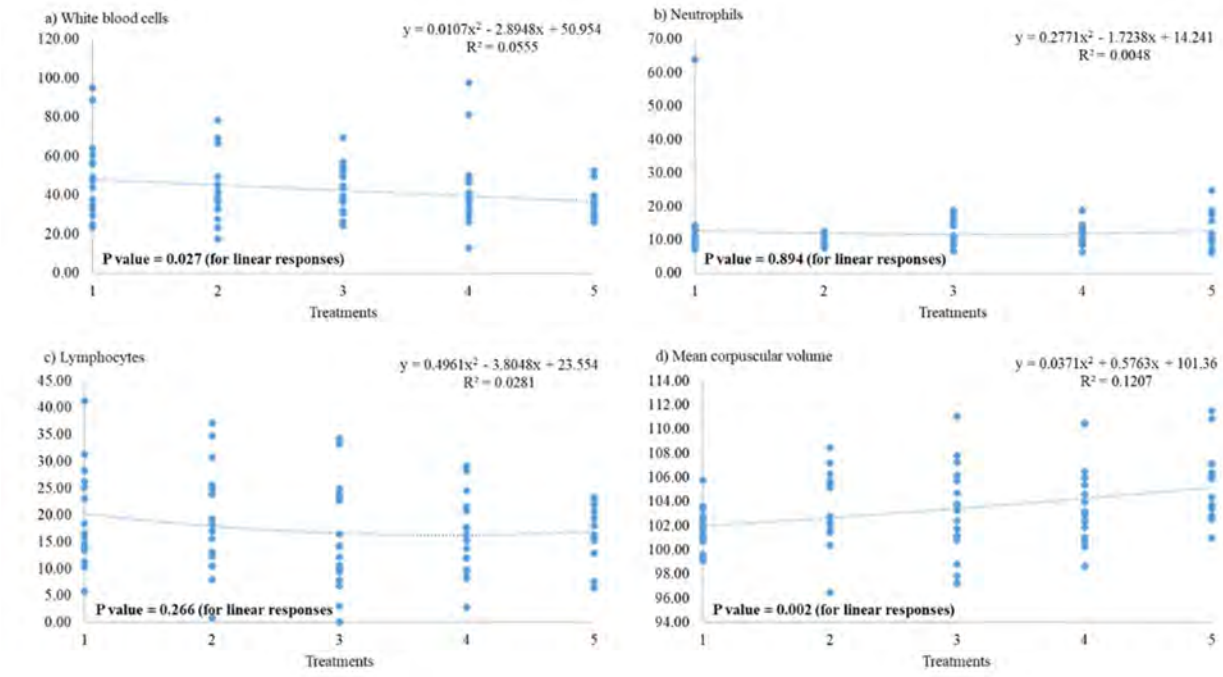


Figure 11 – Total blood count 42 days old broilers fed with BSF larvae with different inclusion levels (5%, 10%, 15% and 20%).

6.3.2 Intraepithelial lymphocytes results

Flow cytometric analysis was conducted to determine the population changes of the intestinal T lymphocytes according to the level of BSF inclusion (Figure 12). The results show that when the BSF larvae were included in the diets, CD3+ T lymphocytes reduced in the intestine of the birds (Figure 12c). The maximum decrease (fourfold decrease) in CD3 + T lymphocytes was observed between T1 (control diet with no BSF larvae inclusion) and T5 (maximum inclusion level of BSF larvae inclusion – 20%). Examination of the intestinal CD3+ T lymphocyte subpopulations revealed that in control broilers, the major intestinal T lymphocyte subtype was the CD3+ and CD8+ cytotoxic T lymphocytes (approximately 58%), followed by CD3+, CD4+, and CD5+ subtypes (13%) and CD3+ and CD4+ T helper lymphocytes (10%). There was a significant (P=0.016) decrease in the subpopulations of CD3+ T lymphocytes (CD3+, CD8+ cytotoxic intestinal T lymphocytes), which accounted for 20.5% of the observed variance. Broilers fed a 20% BSF larval supplemented diet had a 9.7-fold decrease in CD3+, CD8+ lymphocytes in their intestines compared to birds that were fed a control diet (Figure 13D). Similarly, there was a 5.7-fold significant (P=0.004; 28.3% of the variance) decrease in CD3+, CD4+ and CD8+ T lymphocytes in the intestines of birds fed a 20% larval diet compared to birds fed the control diet (Figure 12f). These data suggested that there were decreases in the intestinal cytotoxic T lymphocyte and CD4+, CD8+ double-stained T lymphocyte populations associated with BSF larvae inclusion in the feed of chickens. In contrast, there were no changes in the intestinal T helper (CD3+, CD4+) lymphocyte populations in broilers fed with BSF larvae.

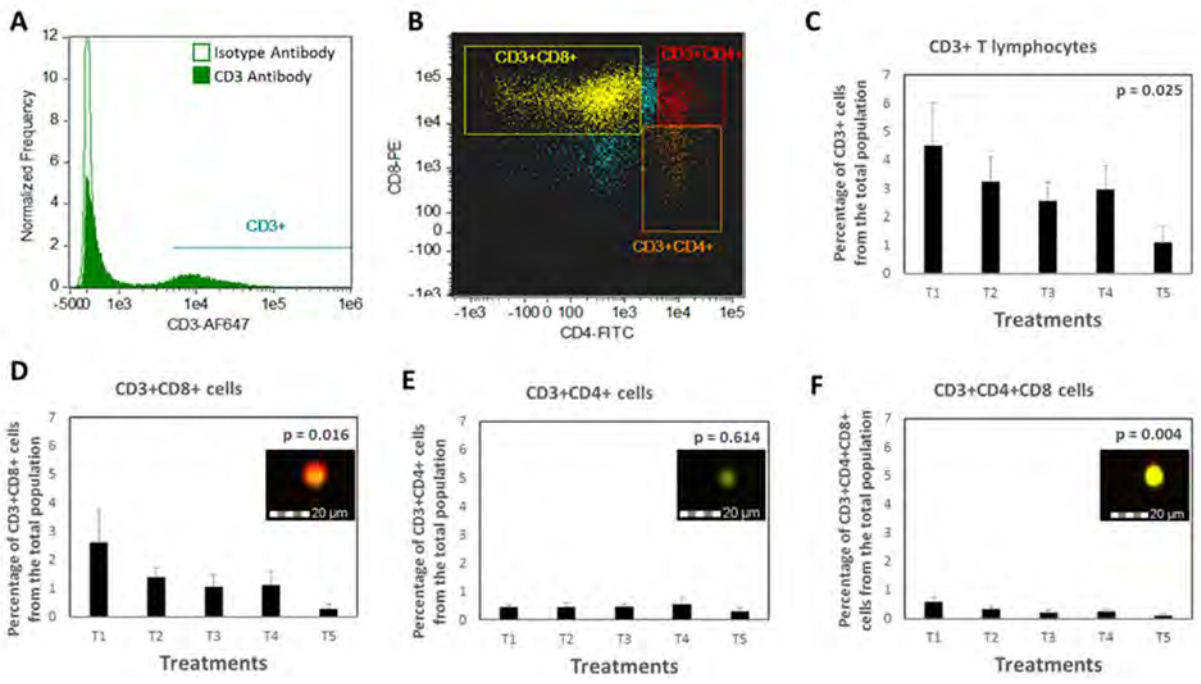


Figure 12 – Flow cytometric analysis of intestinal CD3+ (T-lymphocytes) from chickens given up to 20% BSF larvae in their diets.

7.5 Caecal microbiota

7.5.1 Microbiota profiling

Assessment of the rarefaction curves based on Chao 1 biodiversity indexes calculated indicated that the curves tend to reach a plateau, therefore, in all cases the obtained sequencing data was adequate to cover the vast majority of biodiversity contained within the samples (Figure 13). Further results are presented in Figures 14-19.

7.5.2 Differences in microbial population between treatment groups

Microbiota community profiles of samples were grouped by hierarchical clustering and ordinated by hierarchical clustering and ordinated by Principal Component analysis (PCA). There was no significant difference in any of the treatment groups in both Operational Taxonomic Units (OTUs) and genus level in the microbiome composition as shown in Figure 13. No differences in richness of microbiome at genus level in the microbiome composition as shown in Figure 14. Similarly, there was no difference in the relative abundance (evenness) between the treatment groups.

However, there was a significant difference in the abundance of Enterococcus and Christensenellaceae bacteria (p=0.048; 0.025 respectively) between different treatment groups and the control group as shown in Figure 18. The Dehalobacterium abundance significantly increased with the concentration of the black soldier fly larvae inclusion. Bacteria of *Christensenellaceae* family (phylum Firmicutes) and genus Dehalobacterium has been associated with a high body mass index in humans where lower abundance of these bacteria are associated with a high body mass index (Fu et al. 2015). The impact of this findings on poultry remains unknown to date and needs further investigation.

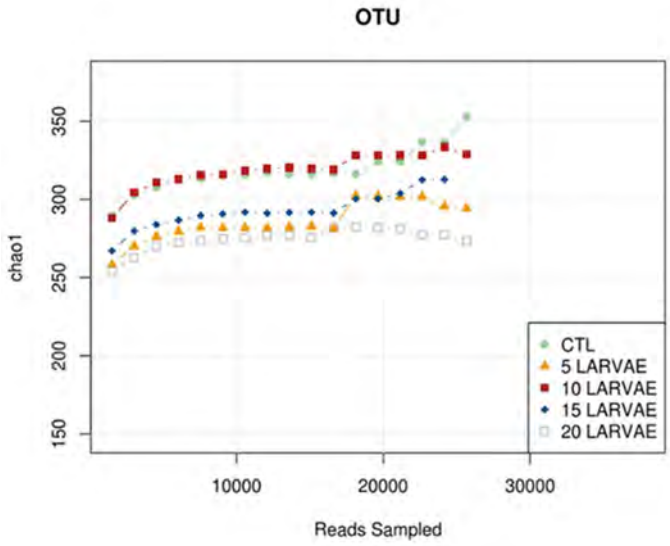


Figure 13: Average rarefaction measure representing variation of the Chao1 diversity (alpha diversity) index of the microbiota of control and four treatment groups; 5%, 10%, 15% and 20% BSF larvae inclusion in broiler feed, respectively corresponding to treatment 1, 2, 3, 4 and 5.

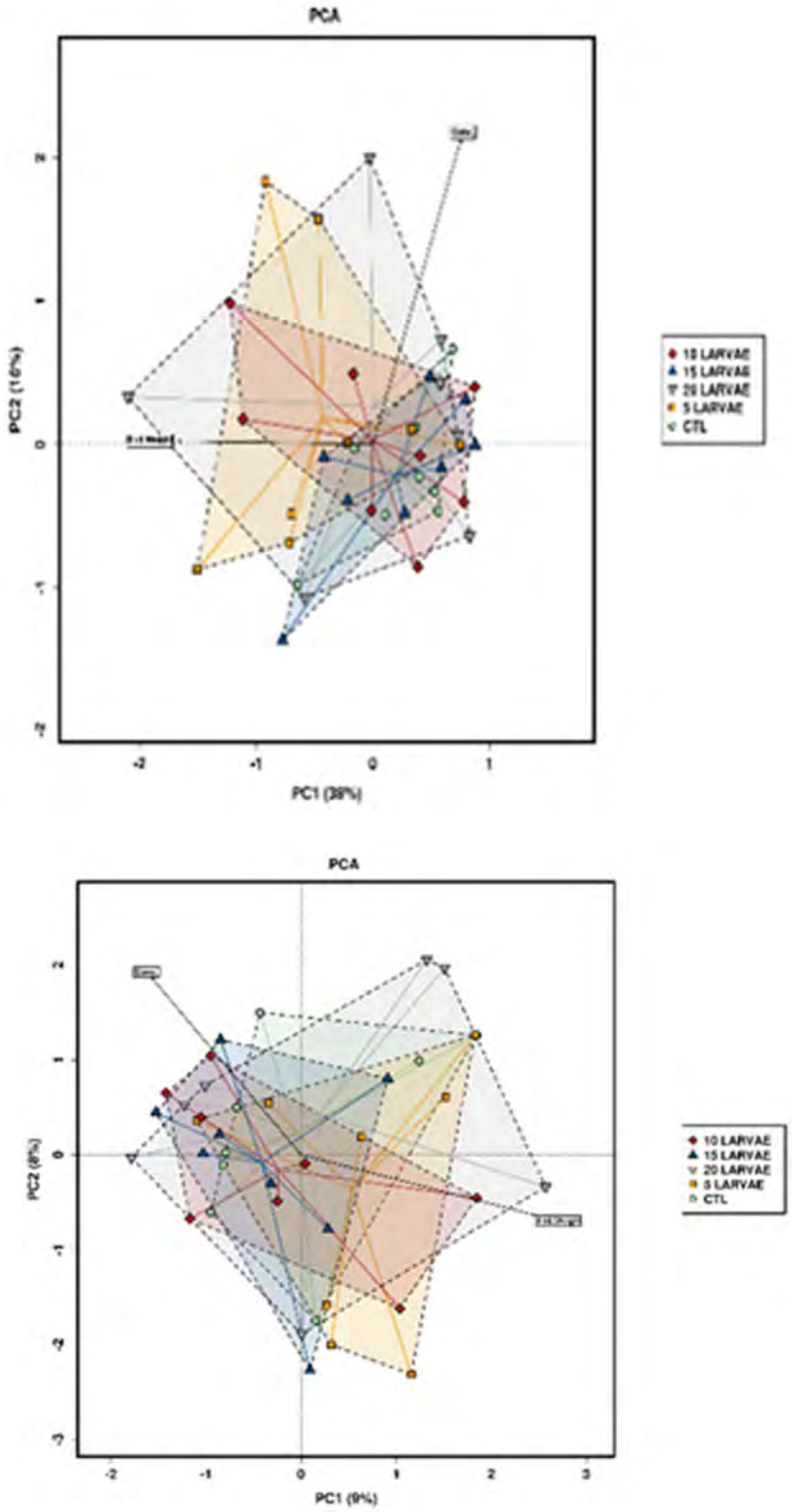


Figure 14: The microbial composition ordered by Principle Component Analysis (PCA) between the control and four treatment groups; 5%, 10%, 15% and 20% BSF larvae inclusion in broiler feed, respectively corresponding to treatment 1, 2, 3, 4 and 5.

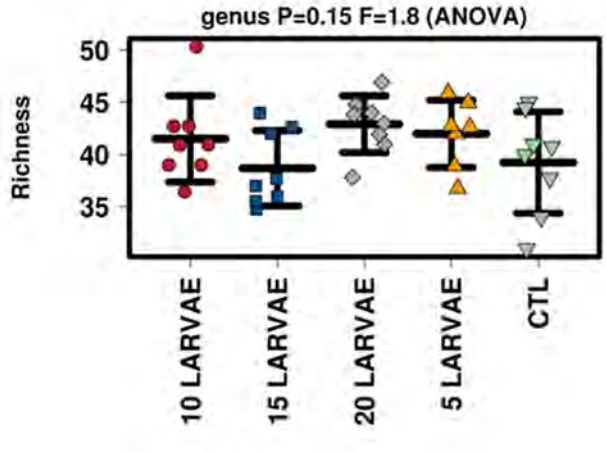


Figure 15: Diversity richness in genus level between the control and four treatment groups; 5%, 10%, 15% and 20% BSF larvae inclusion in broiler feed, respectively corresponding to treatment 1, 2, 3, 4 and 5.

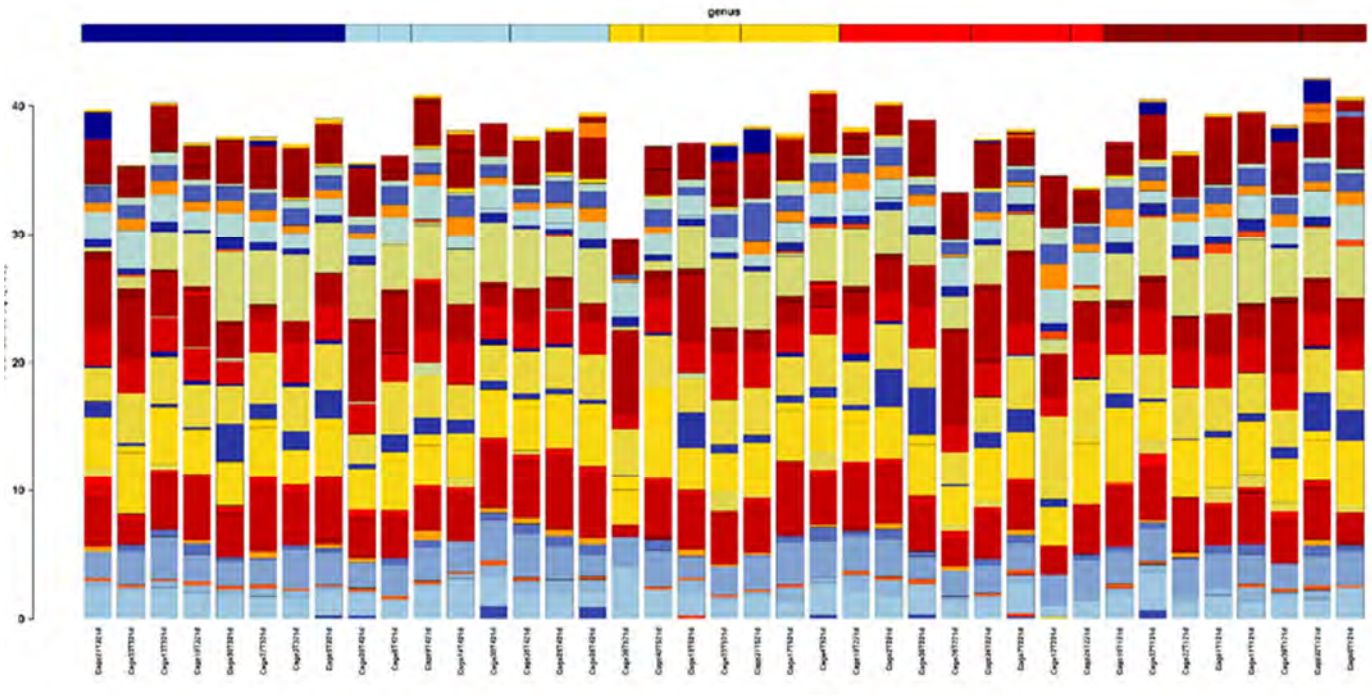


Figure 16: Clustered bar chart within treatment group depicting the relative abundance of microbiome in the treatment group fed with different levels of BSF larvae; 5%, 10%, 15% and 20% in broiler feed, respectively corresponding to treatment 1, 2, 3, 4 and 5.

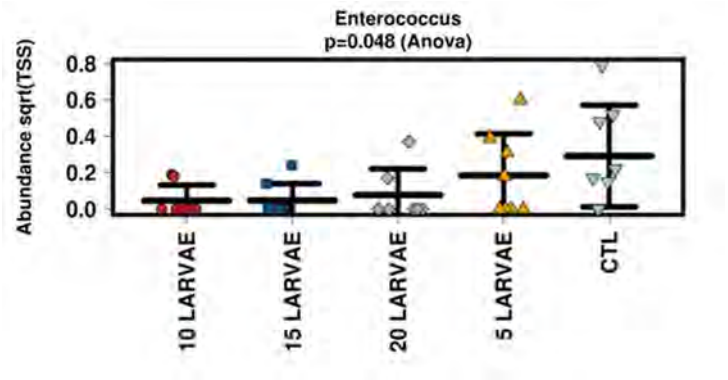


Figure 17: Abundance of Enterococcus between the control and four treatment groups; 5%, 10%, 15% and 20% inclusion level of BSF larvae, respectively corresponding to treatment 1, 2, 3, 4 and 5.

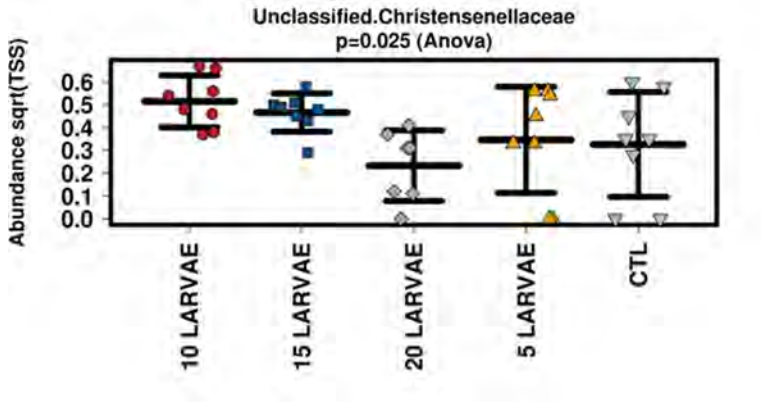


Figure 18: Abundance plot of Christensenellaceae between control and four treatment groups; 5%, 10%, 15% and 20% inclusion level of BSF larvae in broiler feed, respectively corresponding to treatment 1, 2, 3, 4 and 5.

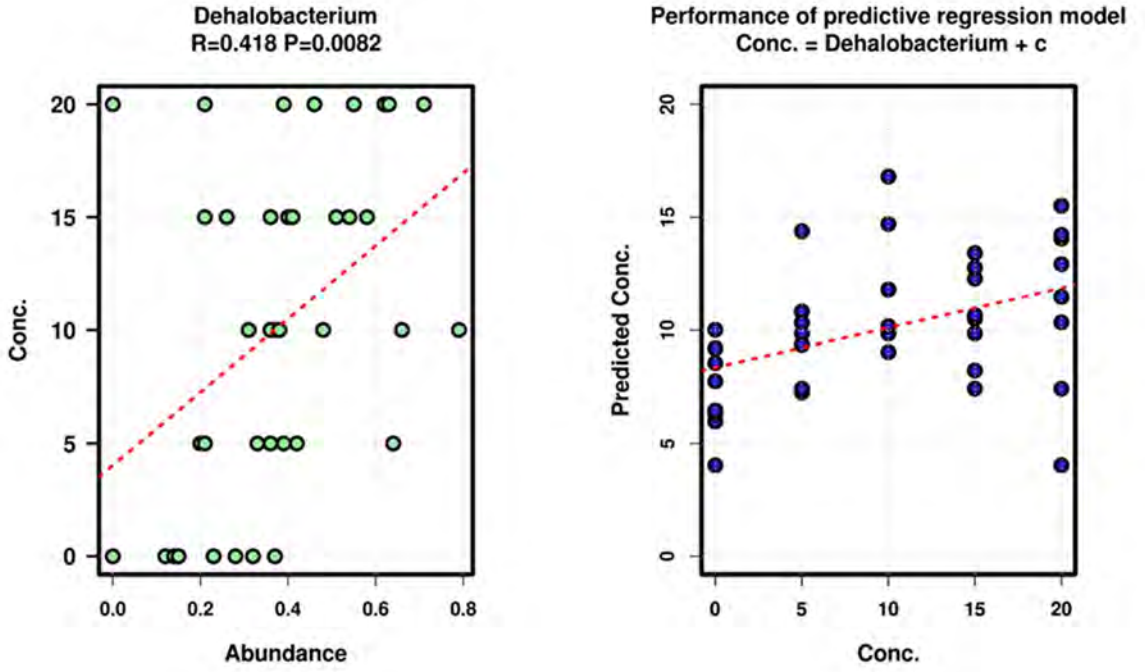


Figure 19: Correlation analysis of the abundance of Dhalobacterium with the concentration of black soldier fly larvae in broiler feed and the predictive regression model.

7.6 Meat quality results

7.6.1. Colour, cooking loss, shear force, and lipid oxidation results

None of the treatment groups including maximum of 20% inclusion of BSF larvae affected the meat quality parameters such as pH, colour, cooking loss, shear force and lipid oxidation (Table 3). Thi sis in agreement with is in line with the studies from Bovera et al. (2015) and Loponte et al. (2017) who showed no negative effects on broilers' growth performance, carcass and meat traits due to addition of insect meal (100% and 25 and 50% inclusion level respectively). Despite the shortage of studies on insects affecting broiler meat quality, the results of this study are similar to others found in the literature. For instance, Schiavone et al. (2016) replaced 100% of soybean oil with BSF larvae fat and none of the color or pH parameters were affected. Cullere et al. (2016), included up to 15% of BSF larvae in quail diets and reported an increase in cooking loss percentage as well as a decrease in redness (a*) between 10 and 15% BSF larvae inclusion treatments, but no difference in quail breast meat colour when comparing treatments with the control. Borgogno et al. (2017), replaced maximum of 50% of fish meal in rainbow trout diets and found no difference in cooking loss or shear force in the rainbow trout meat. Differently from these results, Gasco et al. (2019), replaced soybean oil with BSF larvae and Yellow Mealworm fat in rabbit's diets and reported a reduction in lipid oxidation in rabbit's meat.

Table 3

The effect of the BSF larvae inclusion on meat quality parameters

Item	Treatments						*P-value*
		T1[2]	T2[3]	T3[4]	T4[5]	T5[6]	
pH	Mean	5.70	5.80	5.80	5.70	5.70	0.225
	SEM[1]	±0.10	±0.13	±0.12	±0.09	±0.11	
Color							
L*	Mean	58.2	59.4	58.7	59.0	59.4	0.480
	SEM[1]	±2.94	±2.35	±1.94	±2.88	±2.96	
a*	Mean	4.60	4.50	4.50	4.50	4.20	0.595
	SEM1	±0.89	±1.09	±0.87	±0.78	±0.91	
b*	Mean	0.40	0.70	0.70	0.70	0.90	0.413
	SEM[1]	±0.78	±0.92	±0.68	±1.06	±0.83	
Cooking loss, %	Mean	28.8	28.0	27.9	27.6	28.0	0.751
	SEM	±3.05	±3.67	±2.96	±2.68	±3.41	
Shear force, N	Mean	21.8	20.5	21.5	21.4	24.0	0.628
	SEM[1]	±7.83	±7.17	±6.24	±6.96	±10.3	
Lipid oxidation, mg MDA/kg	Mean	0.70	0.80	0.70	0.70	0.70	0.500
	SEM[1]	±0.07	±0.09	±0.08	±0.07	±0.04	

(L* = lightness, a* = redness, b* yellowness)
[1] SEM = standard error of the mean
[2]T1= Treatment 1 = control group
[3]T2 = Treatment 2 = 2.5% of BSF larvae inclusion until day 10 of the broilers and 5% BSF larvae inclusion from day 11 to day 42
[4]T3 = Treatment 2 = 5% of BSF larvae inclusion until day 10 of the broilers and 10% BSF larvae inclusion from day 11 to day 42
[5]T4 = Treatment 2 = 7.5% of BSF larvae inclusion until day 10 of the broilers and 15% BSF larvae inclusion from day 11 to day 42
[6]T5 = Treatment 2 = 10% of BSF larvae inclusion until day 10 of the broilers and 20% BSF larvae inclusion from day 11 to day 42

7.6.2 Amino acid profile results

There were no differences in the amino acids; histidine, serine, arginine, glycine, aspartic acid, glutamine, threonine, alanine, proline, lysine, tyrosine, methionine, valine, isoleucine, leucine, phenylalanine content, nor the total amount of amino acids in chicken breast samples when up to 20% of larvae was added to broiler chicken diets (Table 4). In contrast, Cullere et al (2018), replaced soybean meal in broiler quails with up to 15% of BSF larvae inclusion and reported increases in the contents of aspartic acid, glutamic acid, alanine, serine, tyrosine and threonine in quail's meat. Further research is warranted as there is a significant lack of knowledge related to the effects of insect inclusion in poultry feed on the meat amino acid composition of the meat obtained for human consumption.

Table 4

Composition of broiler breast meat fed with various levels of BSF larvae

	Means						
Amino acid	T1[1]	T2[2]	T3[3]	T4[4]	T5[5]	SEM[6]	*P - value*
Histidine	24.2	24.2	23.9	23.6	24.2	0.17	0.84
Serine	33.7	33.5	33.6	33.5	33.5	0.15	0.98
Arginine	54.1	53.8	54.0	53.9	53.8	0.25	0.99
Glycine	37.5	37.5	37.5	37.1	37.2	0.15	0.93
Aspartic acid	71.0	71.5	69.8	73.2	72.3	0.46	0.14
Glutamine	118.8	118.5	117.0	121.4	119.2	0.64	0.20
Threonine	37.8	37.7	37.8	37.9	37.8	0.18	1.00
Alanine	44.7	44.8	44.3	45.2	45.0	0.20	0.57
Proline	30.1	30.0	29.9	30.0	30.0	0.12	0.99
Lysine	71.0	71.1	70.1	72.6	71.5	0.39	0.26
Tyrosine	28.0	27.8	28.1	27.9	27.9	0.13	0.99
Methionine	24.1	24.0	24.0	24.1	24.0	0.12	1.00
Valine	41.7	41.7	41.7	42.0	42.0	0.20	0.98
Isoleucine	40.8	40.6	40.7	41.0	40.9	0.19	0.98
Leucine	67.6	67.4	67.3	67.8	67.6	0.30	0.98
Phenylalanine	33.8	33.6	33.8	33.7	33.8	0.16	1.00
Total	759.0	757.7	753.3	764.9	760.7	3.19	0.79

[1]T1= Treatment 1 = control group

[2]T2 = Treatment 2 = 2.5% of BSF larvae inclusion until day 10 of the broilers and 5% BSF larvae inclusion from day 11 to day 42

[3]T3 = Treatment 2 = 5% of BSF larvae inclusion until day 10 of the broilers and 10% BSF larvae inclusion from day 11 to day 42

[4]T4 = Treatment 2 = 7.5% of BSF larvae inclusion until day 10 of the broilers and 15% BSF larvae inclusion from day 11 to day 42

[5]T5 = Treatment 2 = 10% of BSF larvae inclusion until day 10 of the broilers and 20% BSF larvae inclusion from day 11 to day 42

[6]SEM = Standard error of the mean

7.6.3 Fatty acid profile

The BSF larvae had lauric acid as its predominant fatty acid in its composition (Table 2), ~128g/kg out of the total amount of fatty acids in the larvae (~250g/kg) indicating that the BSF larvae used in this study had over 50% of its total amount of fatty acid as lauric acid. In this study, lauric acid (12:0) in breast meat increased from 12.2mg/kg in treatment 1 (5% BSF fly larvae inclusion) to 268.8 mg/kg in treatment 5 (20% of BSF larvae inclusion), consequently also increasing levels of saturated fatty acid (Table 5).

Although the levels of polyunsaturated fatty acids (PUFA) decreased, Eicosapentaenoic acid (EPA, ω3) increased significantly. Usually, an increased concentration of PUFA will increase lipid oxidation. In this study, increased levels of BSF larvae resulted in an increase of EPA without compromising lipid oxidation, which would therefore not impact the rancidification of the product. Partially consistently with these results, Cullere et al. (2019) replaced up to 100% of BSF larvae oil in broiler diets and observed an increase in saturated fatty acids and a decrease in PUFA, having an increase of 11-fold in lauric acid comparing the BSF larvae treatment with the control, but no increase in EPA. Apparently, the composition of the larvae had a high influence in the meat composition and the observed increase of saturated fatty acids may improve sensory meat attributes, such as taste. Further research on this subject would be beneficial.

Table 5
Fatty acids composition (mean ±SEM) of chicken breast from broiler chickens fed different levels of Black Soldier Fly (BSF) larvae:

Fatty acid concentration (mg/100g)	T1	T2	T3	T4	T5	*P - value*
C8:0	5.9±2.7	6.3±2.2	6.4±1.6	6.5±1.7	6.9±1.8	0.671
C10:0	5.8^{b}±2.4	7.3^{b}±2.04	7.9^{ab}±1.6	10.0^{a}±2.8	9.9^{a}±2.6	0.000
C11:0	1.4±0.4	1.1±0.2	1.1±0.4	1.3±0.4	1.4±0.5	0.167
C11:1n - 1	0.5±0.2	0.6±0.3	0.41±0.2	0.42±0.2	0.61±0.4	0.220
C12:0	12.2^{c}±39.0	81.4^{cb}±44.0	139.9^{b}±64.6	221.4^{a}±125.2	268.8^{a}±83.6	0.000
C14:0	18.7^{d}±15.4	42.9^{cd}±19.4	62.3^{cb}±24.7	88.0^{ab}±44.5	103.0^{a}±26.5	0.000
Iso - C15:0	0.1^{c}±0.05	0.2^{b}c±0.1	0.2^{bc}±0.1	0.4^{ab}±0.3	0.5^{a}±0.2	0.000
c14:1n-5	1.4^{c}±1.3	2.9^{bc}±1.3	5.5^{b}±2.2	8.6^{a}±4.9	11.5^{a}±3.8	0.000
C16:0	474.9±141.8	511.9±196.8	458.7±144.2	454.9±199.6	417.6±86.5	0.569
C16: 1n - 7t	0.3^{b}±0.11	0.4^{b}±0.16	0.4^{ab}±0.14	0.6^{a}±0.4	0.6^{a}±0.17	0.000
C16: 1n - 7	30.7^{c}±11.8	43.6^{bc}±19.6	54.2^{ab}±18.6	65.7^{ab}±36.0	70.4^{a}±22.4	0.000
iso - C17:0	0.4^{c}±0.2	0.6^{bc}±0.2	0.9^{b}±0.3	1.3^{a}±0.7	1.7^{a}±0.55	0.000
C17:0	4.0±1.6	4.2±2.25	3.9±1.3	3.9±1.2	4.0±0.9	0.972
C16:2n - 4	0.5^{a}±0.1	0.4^{ab}±0.2	0.4^{ab}±0.16	0.3^{bc}±0.17	0.2^{c}±0.10	0.000
C17: 1n - 7	6.2^{a}±1.2	5.3^{ab}±1.7	5.4^{ab}±1.4	5.6^{ab}±0.6	4.9^{b}±0.62	0.050
C16:3n - 4	0.5±0.3	0.3±0.3	0.3±0.2	0.3±0.2	0.3±0.2	0.103
C18:0	213.0^{a}±55.7	214.9^{a}±68.1	176.6^{ab}±49.8	160^{b}±44.9	145.9^{b}±19.4	0.000
C18: 1n - 9t	4.4^{ab}±1.0	5.1^{a}±1.7	3.1^{c}±1.0	3.1^{c}±0.91	3.4^{bc}±0.6	0.000
C18: 1n - 7t	2.9^{a}±2.1	2.4^{ab}±1.1	1.8^{ab}±0.8	1.6^{b}±1.1	1.6^{b}±0.7	0.012
C18: 1n - 9	432.8±137.0	451.5±183.3	421.8±137.6	415.1±177.6	382.9±90.0	0.765
C18: 2n - 6	564.7±176.6	557.5±205.4	488.1±159.1	415.8±177.6	382.9±90.0	0.101
C19: 1n - 12	0.14±0.06	0.12±0.04	0.13±0.05	0.11±0.03	0.11±0.02	0.043
C18: 3n - 6	4.1±1.6	4.0±2.2	3.6±1.2	3.3±1.5	2.9±0.6	0.156

C18: 3n - 4	0.5[a]±0.2	0.4a[b]±0.2	0.4[ab]±0.1	0.3[bc]±0.1	0.2[c]±0.05	0.000
C18: 3n - 3	19.1±6.9	19.8±9.3	18.6±6.6	19.3±9.5	17.8±3.8	0.955
C20: 0	2.8[a]±0.6	2.7[ab]±0.8	2.2[bc]±0.5	2.1[c]±0.5	1.9[c]±0.2	0.000
C20: 1n - 15	0.3±0.1	0.3±0.1	0.4±0.1	0.3±0.1	0.3±0.01	0.324
C18: 4n - 3	0.7±0.3	0.7±0.3	0.9±0.3	0.8±0.3	0.8±0.1	0.498
C20: 1n - 12	0.6±0.3	0.7±0.4	0.5±0.2	0.7±0.3	0.7±0.3	0.291
C20: 1n - 9	5.4±2.0	5.7±2.0	5.2±1.4	5.2±2.1	5.1±0.8	0.809
C 18: 4n - 1	0.5±0.5	0.5±0.4	1.0±1.3	1.2±1.3	1.4±1.8	0.138
C22: 6n - 3	6.5±1.2	5.5±1.3	5.7±1.1	5.6±1.4	5.7±1.2	0.205
C24: 1n - 9	1.6[b]±0.2	1.6[b]±0.2	1.7[ab]±0.2	1.9[a]±0.2	1.9[a]±0.3	0.005
C22: 5n - 6	4.5[a]±1.3	4.0[ab]±1.2	3.9[ab]±1.1	3.1[bc]±1.0	2.4[c]±0.5	0.000
C22: 5n - 3	8.8±1.0	8.4±1.5	8.5±1.1	8.4±1.2	8.4±1.5	0.862
C23:0	0.4±0.1	0.3±0.1	0.4±0.1	0.3±0.1	0.3±0.05	0.384
C22:0	1.4[a]±0.2	1.4[ab]±0.2	1.2[b]±0.2	1.2[b]±0.2	1.2[b]±0.1	0.000
C20: 3n - 6	10.6±1.8	10.9±1.5	10.5±1.6	10.5±1.7	10.6±1.6	0.971
C20: 3n - 3	1.0±0.1	1.0±0.1	1.0±0.2	1.0±0.2	1.0±0.1	0.909
C20: 4n - 6	59.2[a]±4.7	55.0[ab]±7.1	56.9[ab]±8.4	51.4[bc]±7.6	45.6[c]±6.8	0.000
C20: 3n - 9	1.3[c]±0.3	1.6[bc]±0.4	1.5[bc]±0.3	1.7[b]±0.4	2.1[a]±0.4	0.000
C20: 2n - 6	13.5[a]±2.2	12.9[ab]±1.7	11.1[bc]±2.3	10.0[cd]±1.6	8.2[d]±1.4	0.000
C21:0	1.5±0.5	1.7±0.7	1.7±0.5	1.7±0.7	1.7±0.4	0.685
C22: 1n - 9	0.4±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.04	0.431
C20: 5n - 3	1.4[c]±0.3	1.5[c]±0.3	1.6[bc]±0.3	1.9[b]±0.3	2.5[a]±0.3	0.000
C22: 2n - 6	0.5±0.1	0.4±0.1	0.4±0.2	0.4±0.1	0.3±0.1	0.006
C22: 4n - 6	16.6[a]±1.9	14.8[ab]±2.0	15.2[a]±2.9	12.8[bc]±1.9	10.9[c]±1.9	0.000
C24: 0	0.9[a]±0.1	0.8[ab]±0.2	0.8[ab]±0.1	0.8[b]±0.1	0.8[ab]±0.1	0.041

[1]T1= Treatment 1 = control group

T2 = Treatment 2 = 2.5% of BSF larvae inclusion until day 10 of the broilers and 5% BSF larvae inclusion from day 11 to day 42

[3]T3 = Treatment 2 = 5% of BSF larvae inclusion until day 10 of the broilers and 10% BSF larvae inclusion from day 11 to day 42

[4]T4 = Treatment 2 = 7.5% of BSF larvae inclusion until day 10 of the broilers and 15% BSF larvae inclusion from day 11 to day 42
[5]T5 = Treatment 2 = 10% of BSF larvae inclusion until day 10 of the broilers and 20% BSF larvae inclusion from day 11 to day 42
[a]Represents the greatest value between means when there is significant difference between treatments
[b]Represents a lower than [a] when there is significant difference between treatments
[c]Represents a value that is lower than [b] when there is significant difference between treatments

7.6.4 Carcass composition (lean and fat ratio)

The inclusion of up to 20% of BSF larvae did not affect the carcass composition of chickens, lean or fat ratio. An increase in total bone and ash can be observed as the BSF larvae increased positively. When the ratio of those parameters were calculated there were no significant differences for any of the parameters (Table 6).

Table 6

Composition of chicken carcasses (lean muscle, muscle, total fat, total protein, total bone and total ash) fed on five increasing levels of BSF larvae.

Parameter	T1[4]	T2[5]	T3[6]	T4[7]	T5[8]	SEM	*P - value*
Slaughter weight (g)	3085	3040.5	3099.8	3201.7	3281.3	29	0.06
Primal weight[1] (g)	2704.4	2638.3	2721.8	2808.3	3150.6	65.6	0.11
Lean muscle[2] (g)	2138.8	2087.1	2155.9	2219.3	2506.8	37.3	0.13
Muscle (g)	1641.4	1603.9	1658.9	1704.5	1966.1	49	0.14
Total fat (g)	910	879.9	896.2	937.9	1004.3	14.6	0.06
Total protein (g)	426.8	417	431.3	443.2	511.2	12.7	0.14
Total bone (g)	153.05[a]	154.42[ab]	166.78[ab]	165.85[ab]	180.25[b]	3.06	0.03
Total ash (g)	122.44[a]	123.53[ab]	132.68[ab]	133.42[ab]	144.2[a]	2.45	0.03
			Ratios[3]				
Lean ratio (%)	0.79	0.79	0.79	0.79	0.79	0.001	0.991
Fat ratio (%)	0.34	0.33	0.33	0.33	0.33	0.001	0.208
Protein ratio (%)	0.16	0.16	0.16	0.16	0.16	0.000	0.572
Muscle ratio (%)	0.61	0.61	0.61	0.61	0.61	0.001	0.572
Ash ratio (%)	0.05	0.05	0.05	0.05	0.05	0.141	0.141

[1]Carcass weight without gut, liver, spleen and bursa

[2]Muscle + intramuscular fat

[3]Parameter divided by the primal weight

[4]T1= Treatment 1 = control group

[5]T2 = Treatment 2 = 2.5% of BSF larvae inclusion until day 10 of the broilers and 5% BSF larvae inclusion from day 11 to day 42

[6]T3 = Treatment 2 = 5% of BSF larvae inclusion until day 10 of the broilers and 10% BSF larvae inclusion from day 11 to day 42

[7]T4 = Treatment 2 = 7.5% of BSF larvae inclusion until day 10 of the broilers and 15% BSF larvae inclusion from day 11 to day 42

[8]T5 = Treatment 2 = 10% of BSF larvae inclusion until day 10 of the broilers and 20% BSF larvae inclusion from day 11 to day 42

[a]Represents the greatest value between means when there is significant difference between treatments

[b]Represents a lower than a when there is significant difference between treatments

8. Implications

The present research demonstrates the feasibility of including up to 20% BSF larvae in broiler diets without compromising bird's performance or relevant changes in nutrient digestibility, meat quality or intestinal microbiota. The use of this feed ingredient in commercial scale poultry production facilities is highly warranted to obtain in-field data as a reference for commercial use. While various insect products can be used for animal nutrition, this project used whole dried, full fat BSF larvae harvested at day 12 of its development status. Different larvae products (eg. larvae meal, defatted larvae, various other larvae fractions) would interfere in the feed formulation and production, possible changing other ingredients and may affect bird's performance or nutrient digestibility.

Further research is required to investigate whether the effects of the BSF larvae on the immune system can have clinical impact e.g. health benefits. This will allow for investigating the potential use of BSF larvae or its active ingredients (chitosan, antibiotic peptides) as a feed additive e.g. health promoter.

9. Recommendations

It can be recommended that full fat the BSF larvae is suitable for broiler nutrition without compromising their performance, health or meat quality. Based on the data obtained in this study in addition with the literature knowledge, further research on the potential beneficial effects of BSF larvae on the immune system should be investigated.

10. Acknowledgments

We acknowledge Feedworks, Karma3, GoTerra, and the entire UNE poultry team as well and the Meat Science Team and Nicholas Andronicos for all in-kind contributions, support and knowledge shared.

11. Media and Publications

11.1 Australian Poultry Science Symposium, 2020

BLACK SOLDIER FLY LARVAE IN MEAT CHICKEN DIETS MODIFIES THE FATTY ACID PROFILE IN CHICKEN BREAST MEAT

J. DE SOUZA VILELA, T.I.R.C. ALVARENGA, D. HOPKINS, M. KOLAKSHYAPATI1, P. MCGILCHRIST, I. RUHNKE

One of the reasons chicken meat is considered healthy food is due to its relatively low-fat level. Black soldier fly (BSF) larvae are a promising alternative feed ingredient for monogastrics, including poultry. However, the impact of feeding BSF larvae in broiler diets on the fatty acid profile of the breast chicken meat remains unknown. This study aimed to investigate the impact of up to 20% BSF larvae dietary inclusion on the fatty acid profile of meat chicken breast meat when fed until 42 days of age.

A total of 400 male day-old Ross 308 birds were assigned to 10 birds/pen, five treatment groups with eight replicates per treatment. The five dietary treatments included increasing levels of BSF larvae as follows: the starter diets (fed from day 2 -10) included 0, 2.5, 5, 7.5 and 10% BSF larvae, whereas, the grower (fed from day 11-21) and finisher diets (fed from day 22-42) included 0, 5, 10, 15 and 20% BSF larvae. All diets were isocaloric and met breeder's nutrient recommendations. At 42 days of age, two birds per treatment group were euthanised, and their breast meat immediately removed, placed in a plastic bag, and stored overnight at 4°C. On the following day, ~40g of meat was weighed and kept at -20 °C until lyophilisation and grinding. Thereafter the fatty acid profile of the 80 chicken breasts were analysed along with lyophilised BSF larvae using a fused carbon–silica column coated with cyanopropylphenyl (BPx70, 30mx 0.25 mm id and 0.25µm thickness, SGE Analytical Science, Ring Wood, Victoria, Australia, P/N 054622) and gas chromatography according to the method described by Clayton et al., 2012. A total of 48 fatty acids were identified in the breast meat samples, and data were analysed using IBM SPSS version 25 to conduct a curve estimation regression for linear, cubic, and quadratic responses. As a result, there was no significant difference among the groups in the total saturated (SFA) or total monounsaturated (MUFA) fatty acids, but individual linear increases ($P < 0.001$) were observed for SFA such as lauric acid and myristic acid, as well as a linear decrease ($P < 0.001$) in stearic acid as the BSF larvae level increased in the diets. A significant negative correlation reflected a linear decrease of total polyunsaturated fatty acids (PUFA) in the breast meat with increasing levels of BSF. However, there were no significant changes in linoleic acid (ω6), linolenic acid (ω3), docosapentaenoic acid (ω3) and docosahexaenoic acid (ω3). There was a significant linear increase in eicosapentaenoic acid, EPA (ω3), and mead acid (ω9), as well as a linear decrease in adrenic acid (ω6) with increasing inclusion of BSF. The increase of EPA and mead acid in the chicken breast meat was almost 1 and 0.5-fold, respectively, when 20% of BSF larvae was included in the diets. In conclusion, by including up to 20% BSF larvae in the diet, the concentration of total PUFA in the chicken breast was reduced. However, there were significant increases (1, 0.5, and 22 fold respectively) in EPA (ω3), mead acid (ω9) and lauric acid, which are fatty acids with beneficial properties (e.g. cardiovascular disease risk reduction, anticancer and antimicrobial) and therefore may affect the status of chicken meat as a healthy food for humans.

Clayton EH, Gulliver CE, Piltz JW, Taylor RD, Blake RJ & Meyer RG (2012) Lipids. 47: 719-727.

School of Environmental and Rural Science, Faculty of Science Agriculture Business and Law, University of New England, Armidale NSW 2651, Australia; jdesouz2@myune.edu.au

Livestock Industries Centre, NSW Department of Primary Industries, Armidale NSW 2351, Australia; tharcilla.alvarenga@dpi.nsw.gov.au

Red Meat and Sheep Development Centre, NSW Department of Primary Industries, Cowra NSW 2794, Australia; david.hopkins@dpi.nsw.gov.au

11.2 Recent Advances in Animal Nutrition, 2019

Black soldier fly larvae does not compromise broiler meat quality

Jessica de Souza Vilela[1], Tharcilla I.R.C. Alvarenga[2], Manisha Kolakshyapati[1], Peter McGilchrist[1] and Isabelle Ruhnke[1]

[1]School of Environmental and Rural Science, Faculty of Science Agriculture Business and Law, University of New England, Armidale, Australia
[2] NSW Department of Primary Industries, Centre for Red Meat and Sheep Development, Cowra, NSW, 2794 Australia

The demand for novel commercial protein sources in replacement of soybean and fishmeal in livestock feed has largely increased as a result of a costly soybean and fish meal-based diets. Black soldier fly larvae (BSFl) has demonstrated efficiency in partial or total replacement of soybean and fish meal in broiler diets without compromising bird's performance (Maurer et al., 2015; Vilela et al., 2019). However, the impact of BSFl on meat quality is just as relevant as bird's performance, as the final product (chicken meat) has to be accepted by consumers. Thus, it was hypothesized that the composition of the BSFl would affect meat quality of broiler meat fed on high inclusion levels of BSFl. A total of 400 Ross - 308 broilers were housed in 40 cages allowing for 8 replicates of the 5 treatment groups. Birds were fed with 0, 2.5, 5, 7.5 and 10 % of BSFl during the starter period (2-10 days of age), and 0, 5, 10, 15 and 20% of BSFl during grower and finisher period (11-42 weeks of age). On day 42, 2 birds per cage were randomly selected, humanely killed, and breast meat immediately removed and placed in plastic bags at 4 ℃ overnight. On the following day meat colour was assessed using a Minolta Chroma Meter CR 300 (Minolta Co., Ltd., Japan). The meat cut surface was exposed to the air for 35 minutes, three replicate measurements were taken placing the colorimeter across the fibre position on the bloomed surface of each breast. Colour parameters were set on the L^*, a^*, b^* system where L^* measures lightness, a^* relative redness and b^* relative yellowness. Measurements of pH and temperature were undertaken by a direct insertion of a pH probe (IJ44C probe, Ionode, Pty Ltd., Australia) in the muscle samples. The measurements were taken in duplicate using a digital pH meter with a combination electrode (glass body with a spear tip. A subsample was collected, vacuum packed and frozen at -80 °C for lipid oxidation analysis using TBARS (thiobarbituric acid reactive substance) qualification method (Zhang et al., 2019). Data was analysed as a completely randomised design which included levels of BSFl as fixed effect, using pH as covariate in colour analysis. IBM SPSS version 25 was used to conduct Univariate Analysis of Variance. There was no significant effect of pH as a covariate on colour parameters ($P > 0.05$). Feed treatment had no effect on pH (5.74 ± 0.11), colour (L^* 59.0 ± 0.88; a^* 4.47 ± 0.91 and b^* 0.69 ± 0.88) and lipid oxidation (0.72 ± 0.07 mg MDA/kg) of breast chicken muscle ($P > 0.05$). In conclusion, the inclusion of up to 10% BSFl fed until day 10 of age followed by 20% BSFl in broiler diets fed until 42 days of age had no detrimental effect on colour, pH and lipid oxidation of chicken breast.

Maurer, V, Holinger, M, Amsler, Z, Früh, B, Wohlfahrt, J, Stamer, A (2015) "Replacement of Soybean Cake by Hermetia Illucens Meal in Diets for Layers." *Jounal of Insects of Food Feed* **2**, 83 – 90.

Vilela, J, Andronicos, N, Hilliar, M, Andrew, N, Swick, R, Ruhnke, I (2019) "Black Soldier Fly Larvae in Broiler Diets Did Not Affect Performance but Decreased Cellular Immune Parameters " 22nd European Symposium in Poultry Nutrition (ESPN 2019), Gadank – Poland.

Zhang, Y, Benjamin, W. B. H, Eric, N. P, Matthew, G. K, Kristy, L. B, Ashleigh, K. K, Damian, C, David L. H (2019) "Understanding Beef Flavour and Overall Liking Traits Using Two Different Methods for Determination of Thiobarbituric Acid Reactive Substance (TBARS)." *Meat Science* **149**: 114-19.

11.3 Presentation for PHA Ideas Exchange, 2019

The Black Soldier Fly Larvae in Poultry Feed

Presenter: Jessica de Souza Vilela
Supervisors: Isabelle Ruhnke, Nigel Andrew, Robert Swick
Industry collaborators: David Cadogan, Stuart Wilkinson, Olympia Yarger, Alex Arnold

Background/Introduction

- Importations of soy ↑ 80% (2000 – 2019)
- Greatest barrier: Natural Resources
- The Black Soldier Fly larvae ↓ water usage
- Up to 40% CP
- 37% Fat

Project Aims

- Determine maximum inclusion level of the Black Soldier Fly larvae in broiler feed;
- Determine effect on:

Performance | Digestibility | Immunity | Meat quality | Microbiota

- Allows BSF use in the future

Methodology

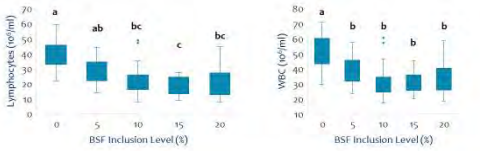
- 400 Ross 308 male broiler chickens
- 10 birds/cage, 8 replicates/treatment
- Statistical analysis
  - One-way ANOVA
  - Orthogonal Polynomial Regression

	Larvae inclusion levels, %				
	T1	T2	T3	T4	T5
Starter diet	0	2.5	5	7.5	10
Grower diet	0	5.0	10	15	20
Finisher diet	0	5.0	10	15	20

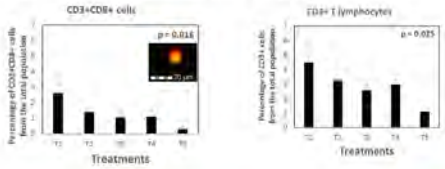
Impact

- BSF larvae can be included up to 20% in broiler diets
- No compromise on bird performance
- No compromise on meat quality
- Modulate broiler immune parameters

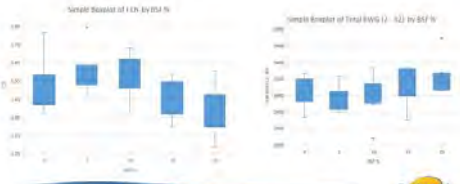
BSF - Blood cells



BSF - Intraepithelial lymphocytes



Take Home Message



Take Home Message

- Up to 20% BSF can be used in broiler diets
- Alternative sustainable feed ingredient with potential health benefits

11.4 European Symposium of Poultry Nutrition, 2019

Black Soldier Fly Larvae in broiler diets did not affect performance but decreased cellular immune parameters

Jessica de Souza Vilela[1], Nick Andronicos[3], Matthew Hilliar[1], Nigel Andrew[2], Robert Swick[1] and Isabelle Ruhnke[1]

To evaluate the effects of different inclusion levels of dried Black Soldier Fly larvae (BSF) in broiler diets, five inclusion levels of BSF were investigated in the starter diets (0, 2.5, 5, 7.5 and 10%), and the grower and finisher diets (0, 5, 10, 15 and 20%). Diets were fed to a total of 400 broilers, placed in cages with 8 replicates per treatment. For the starter (2d to 10d), there was no significant difference in total broiler performance across the treatments. However, during the grower period (11d to 21d), means feed intake (FI), body weight (BW), and feed conversion ratio (FCR) were significantly different across five treatments (ANOVA, $P < 0.05$); broilers with 10% larvae inclusion diet had the highest FCR and the highest FI. Furthermore, orthogonal polynomial analysis reveals that the FI has a negative quadratic response to graded levels of BSF inclusion ($P < 0.05$). Means white blood cell and lymphocytes were also significantly different in broilers across five treatments (ANOVA test $P < 0.05$). Those birds with 15% and 20% larvae diets had significantly lower lymphocytes and white blood cell count compared to the control group (Tukey post hoc test, $P < 0.001$). In the finisher period, there was a significant ($P < 0.05$) four-fold decrease in cluster of differentiation 3 (CD3+) lymphocytes and a significant 9.7 fold decrease in the population of CD3+CD8+ intestinal cytotoxic T lymphocytes in birds fed a 20% BSF larval diet compared to the control group. These findings suggest that inclusion of BSF may improve performance and affect the immune status in broilers.

KEYWORDS: Alternative Protein Source, Growth Performance, Insects, Poultry, Production

[1]School of ERS, Faculty of SABL, University of New England, Armidale, Australia
[2]Insect Ecology Lab, Zoology, University of New England, Armidale, Australia
[3]Biomedical Sciences, School of Science and Technology, University of New England, Armidale, Australia

12. Intellectual Property Arising

N/A

13. References

Altmann, B. A., C. Neumann, S. Velten, F. Liebert and D. Mörlein (2018). "Meat Quality Derived from High Inclusion of a Micro-Alga or Insect Meal as an Alternative Protein Source in Poultry Diets: A Pilot Study." Foods 7(3): 34.

Arnold van Huis and Jeffery K. Tomberlin (2017). Insects as food and feed from production to consumption The Netherlands Wageningen Academic

Banks, I. J., W. T. Gibson and M. M. Cameron (2014). "Growth rates of black soldier fly larvae fed on fresh human faeces and their implication for improving sanitation." Tropical Medicine & International Health 19(1): 14-22.

Beskin, K. V., C. D. Holcomb, J. A. Cammack, T. L. Crippen, A. H. Knap, S. T. Sweet and J. K. Tomberlin (2018). "Larval digestion of different manure types by the black soldier fly (Diptera: Stratiomyidae) impacts associated volatile emissions." Waste Management 74: 213-220.

Bram Dortmans (2017). "BSF_Biowaste_Processing_LR.pdf." Sandec: department of sanitation, water and solid waste for development: 1 - 100.

Borgogno, M., C. Dinnella, V. Iaconisi, R. Fusi, C. Scarpaleggia, A. Schiavone, . . . G. Parisi. Inclusion of Hermetia illucens larvae meal on rainbow trout (Oncorhynchus mykiss) feed: effect on sensory profile according to static and dynamic evaluations. J Sci Food Agric 2017. 97 (10):3402-11.

Cheng, J. Y. K., S. L. H. Chiu and I. M. C. Lo (2017). "Effects of moisture content of food waste on residue separation, larval growth and larval survival in black soldier fly bioconversion." Waste Management 67: 315-323.

Cullere, M., G. Tasoniero, V. Giaccone, G. Acuti, A. Marangon, and A. Dalle Zotte. Black soldier fly as dietary protein source for broiler quails: Meat proximate composition, fatty acid and amino acid profile, oxidative status and sensory traits. Animal 2018. 12 (3):640-47.

Cullere, M., G. Tasoniero, V. Giaccone, R. Miotti-Scapin, E. Claeys, and S. Smet. Black soldier fly as dietary protein source for broiler quails: apparent digestibility, excreta microbial load, feed choice, performance, carcass and meat traits. Animal 2016. 10.

Cutrignelli, M. I., M. Messina, F. Tulli, B. Randazzo, I. Olivotto, L. Gasco, R. Loponte and F. Bovera (2018). "Evaluation of an insect meal of the Black Soldier Fly (Hermetia illucens) as soybean substitute: Intestinal morphometry, enzymatic and microbial activity in laying hens." Research in Veterinary Science 117: 209-215.

Cullere, M., A. Schiavone, S. Dabbou, L. Gasco, and A. Dalle Zotte. Meat Quality and Sensory Traits of Finisher Broiler Chickens Fed with Black Soldier Fly (Hermetia Illucens L.) Larvae Fat as Alternative Fat Source. Animals 2019. 9 (4):140.

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

Denis M' Gee (2011). Soybeans in the Australian and Global Market 2011 S. A. L. A. Soybeans. URL: http://www.australianoilseeds.com/__data/assets/pdf_file/0005/8177/Industry__and__Market_Review_2011.pdf

Devic, E., W. Leschen, F. Murray and D. C. Little (2018). "Growth performance, feed utilization and body composition of advanced nursing Nile tilapia (Oreochromis niloticus) fed diets containing Black Soldier Fly (Hermetia illucens) larvae meal." Aquaculture Nutrition 24(1): 416-423.

Diener, S., C. Zurbrügg and K. Tockner (2015). "Bioaccumulation of heavy metals in the black soldier fly, Hermetia illucens and effects on its life cycle." Journal of Insects as Food and Feed 1(4): 261-270.

Dumas, A., T. Raggi, J. Barkhouse, E. Lewis and E. Weltzien (2018). "The oil fraction and partially defatted meal of black soldier fly larvae (Hermetia illucens) affect differently growth performance, feed efficiency, nutrient deposition, blood glucose and lipid digestibility of rainbow trout (Oncorhynchus mykiss)." Aquaculture 492: 24-34.

Elieh Ali Komi, D., Sharma, L. and C.S. Dela Cruz. (2018) Chitin and its effects on inflammatory and immune responses. Clin. Rev. Allergy Immunol 2018; 54(2):213-223.

European Union (2017). "Commission regulation (EU) 2017/893." Official Journal of the European Union 138: 93-116.

Folch, J., M. Lees, and G. S. Stanley. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957. 226 (1):497-509.

Fortuoso, B.F., dos Reis, J.H., Gebert, R.R., Barreta, M., Griss, L.G., Casagrande, R.A., de Cristo, T.G., Santiani, F., Campigotto, G., Rampazzo, L., Stefani, L.M., Boiago, M.M., Lopes, L.Q., Santos, R.C.V., Baldissera, M.D., Zanette, R.A., Tomasi, T. and Da Silva, A.S. (2019). "Glycerol monolaurate in the diet of broiler chickens replacing conventional antimicrobials: Impact on health, performance and meat quality. " Microb. Pathog 129: 161-167.

Gasco, L., S. Dabbou, F. Gai, A. Brugiapaglia, A. Schiavone, M. Birolo, A. Trocino. "Quality and Consumer Acceptance of Meat from Rabbits Fed Diets in Which Soybean Oil is Replaced with Black Soldier Fly and Yellow Mealworm Fats. Animals 2019. 9 (9):629.

Lee, J.-A., Y.-M. Kim, Y.-K. Park, Y.-C. Yang, B.-G. Jung and B.-J. Lee (2018). "Black soldier fly (Hermetia illucens) larvae enhances immune activities and increases survivability of broiler chicks against experimental infection of Salmonella Gallinarum." Journal of Veterinary Medical Science doi: 10.1292/jvms.17-0236.

Londok, J and J. Rompis (2019). Supplementation of lauric acid and feed fiber to optimize the performance of broiler. In: IOP Conference Series: Earth and Environmental Science. IOP Publishing. 012082.

Marono, S., R. Loponte, P. Lombardi, G. Vassalotti, M. E. Pero, F. Russo, L. Gasco, G. Parisi, G. Piccolo, S. Nizza, C. Di Meo, Y. A. Attia and F. Bovera (2017). "Productive performance and blood profiles of laying hens fed Hermetia illucens larvae meal as total replacement of soybean meal from 24 to 45 weeks of age." Poultry Science 96(6): 1783-1790.

Maurer, V., M. Holinger, Z. Amsler, B. Früh, J. Wohlfahrt and A. Stamer (2016). "Replacement of soybean cake by Hermetia illucens meal in diets for layers." J Insects Food Feed 2(2): 83 – 90.

Mwaniki, Z., M. Neijat and E. Kiarie (2018). "Egg production and quality responses of adding up to 7.5% defatted black soldier fly larvae meal in a corn–soybean meal diet fed to Shaver White Leghorns from wk 19 to 27 of age." Poultry Science: 118-118.

Nelson DW and LE Sommers. 1982. "Total carbon, organic carbon, and organic matter". In: AL Page, RH Miller and DR Keeney (eds). Methods of Soil Analysis. Part 2 Chemical and Microbiological Properties. American Society of Agronomy - Soil Science
Society of America, Madison, WI, pp. 539-579.

Nguyen, T. T. X., J. K. Tomberlin and S. Vanlaerhoven (2013). "Influence of Resources on Hermetia illucens (Diptera: Stratiomyidae) Larval Development." Journal of Medical Entomology 50(4): 898-906.

Nguyen, T. T. X., J. K. Tomberlin and S. Vanlaerhoven (2015). "Ability of Black Soldier Fly (Diptera: Stratiomyidae) Larvae to Recycle Food Waste." Environmental Entomology 44(2): 406-410.

Ruhnke, I., C. Normant, D. L. M. Campbell, Z. Iqbal, C. Lee, G. N. Hinch and J. Roberts (2018). "Impact of on-range choice feeding with black soldier fly larvae (Hermetia illucens) on flock performance, egg quality, and range use of free-range laying hens." Animal Nutrition. doi.org/10.1016/j.aninu.2018.03.005

Schiavone, A., M. Cullere, M. Marco, M. Meneguz, I. Biasato, and S. Bergagna. 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 2016. 16.

Schiavone, A., M. Cullere, M. De Marco, M. Meneguz, I. Biasato, S. Bergagna, D. Dezzutto, F. Gai, S. Dabbou, L. Gasco and A. Dalle Zotte (2017a). "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 s, carcass characteristics and meat quality." Italian Journal of Animal Science 16(1): 93-100.

Schiavone, A., M. De Marco, S. Martínez, S. Dabbou, M. Renna, J. Madrid, F. Hernandez, L. Rotolo, P. Costa, F. Gai and L. Gasco (2017b). "Nutritional value of a partially defatted and a highly defatted black soldier fly larvae (Hermetia illucens L.) meal for broiler chickens: apparent nutrient digestibility, apparent metabolizable energy and apparent ileal amino acid digestibility." Journal of Animal Science and Biotechnology 8(1): 51-59.

Secci, G., F. Bovera, S. Nizza, N. Baronti, L. Gasco, G. Conte, A. Serra, A. Bonelli and G. Parisi (2018). "Quality of eggs from Lohmann Brown Classic laying hens fed black soldier fly meal as substitute for soya bean." animal: 1-7.

Sheppard, C. (1983). "House Fly and Lesser Fly Control Utilizing the Black Soldier Fly in Manure Management Systems for Caged Laying Hens." Environmental Entomology 12(5): 1439-1442.

Spranghers, T., J. Michiels, J. Vrancx, A. Ovyn, M. Eeckhout, P. De Clercq and S. De Smet (2018). "Gut antimicrobial effects and nutritional value of black soldier fly (Hermetia illucens L.) prepupae for weaned piglets." Animal Feed Science and Technology 235: 33-42.

Spranghers, T., M. Ottoboni, C. Klootwijk, A. Ovyn, S. Deboosere, B. De Meulenaer, J. Michiels, M. Eeckhout, P. De Clercq and S. De Smet (2017). "Nutritional composition of black soldier fly (Hermetia illucens) prepupae reared on different organic waste substrates." Journal of the Science of Food and Agriculture 97(8): 2594-2600.

St-Hilaire, S., K. Cranfill, M. A. McGuire, E. E. Mosley, J. K. Tomberlin, L. Newton, W. Sealey, C. Sheppard and S. Irving (2007). "Fish Offal Recycling by the Black Soldier Fly Produces a Foodstuff High in Omega-3 Fatty Acids." Journal of the World Aquaculture Society 38(2): 309-313.

Tschirner, M. and A. Simon (2015). "Influence of different growing substrates and processing on the nutrient composition of black soldier fly larvae destined for animal feed." J Insects Food Feed 1(4): 249-259.

Vogel, H., Müller, A., Heckel, D.G., Gutzeit, H. and A. Vilcinskas (2018). “Nutritional immunology: Diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly *hermetia illucens*.” Dev Comp Immunol 78:141-148. Wallace, P. A., J. K. Nyameasem, G. A. Aboagye, S. Affedzie-Obresi, K. Nkegbe, F. Murray, V. Botchway, N. Karbo, W. Leschen, P.-O. Maquart and V. Clottey (2018). "Effects of replacing fishmeal with black soldier fly larval meal in the diets of grower-finishing guinea fowls reared under tropical conditions." Tropical Animal Health and Production.1:1-9.

Zheng, A., J. Luo, K. Meng, J. Li, W. L. Bryden, W. Chang, . . . B. Yao. Probiotic (Enterococcus faecium) induced responses of the hepatic proteome improves metabolic efficiency of broiler chickens (Gallus gallus). BMC Genomics 2016. 17 (1):89.