



## Original Research Article

# Black soldier fly larvae in broiler diets improve broiler performance and modulate the immune system



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## ABSTRACT

Non-conventional feed ingredients are receiving more interest in their ability to increase farming efficiency, sustainability and animal performance. The objective of this study was to determine the optimal rate of inclusion level of the full-fat black soldier fly larvae (BSFL) in broiler diets and to evaluate their impact on performance, nutrient digestibility, and the immune system (blood cells and intraepithelial lymphocytes). A total of 400 male day-old Ross 308 broilers were randomly assigned to 5 treatment groups with 8 replicates each. Five inclusion levels of full-fat BSFL were investigated across starter (0, 2.5%, 5%, 7.5% and 10%), grower and finisher diets (0, 5%, 10%, 15% and 20%). All diets were formulated based on digestible amino acid values according to the Aviagen (2016) recommendations. A polynomial regression at different degrees was performed to analyse broiler performance parameters (body weight, body weight gain, feed intake, and feed conversion ratio), nutrient digestibility, and blood cell count. Intraepithelial lymphocyte population data was analysed performing univariate linear regression. During the entire experimental period (from 2 to 42 d), BSFL inclusion levels decreased the feed conversion ratio by 10% in broilers that received 20% BSFL in their diets ( $P < 0.05$ ). Lymphocytes and white blood cell count decreased linearly by 47.7% and 35.9%, respectively, with up to 20% BSFL inclusion ( $P < 0.001$ ). A 4-fold decrease in CD3+ T lymphocytes and a 9.7-fold decrease of CD3+CD8+ intestinal cytotoxic T lymphocytes occurred in broilers fed 20% BSFL compared to the control group. These findings suggest that the inclusion of BSFL can improve broiler performance and potentially reduce immune response energy expenditure in birds fed 20% BSFL for 42 d.

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## 1. Introduction

The use of non-conventional feed ingredients such as insects, microalgae, or even food waste in animal production has the potential to increase farming efficiency and sustainability (De Souza-Vilela et al., 2019; Kojima 2005; Madeira et al., 2017). The prominence of non-conventional feed for production animals is expected to increase because of global warming, reduced availability of water, and the reduction in arable farmland. Black soldier fly larvae (BSFL) are known for their capacity to reduce the amount of organic waste sent to landfill, and are a potential alternative feed ingredient in different monogastric animal diets including poultry, pigs, fish, and dogs (Cutrignelli et al., 2018; Devic et al., 2018; Lei et al., 2019;

Spranghers et al., 2018). The full-fat BSFL can replace up to 15% of the conventional feed ingredients in broiler diets reducing the feed cost by 19% when being compared to Kenyan soybean and fish meal diets; indicating that BSFL may be already a cheaper ingredient option with no adverse effects in broiler production parameters in some countries (Onsongo et al., 2018). Other studies have used BSFL in duck and layer diets with no obvious detrimental effects (Dabbou et al., 2018; De Marco et al., 2015; Gariglio et al., 2019; Ruhnke et al., 2018).

The nutrient composition of BSFL makes it an attractive ingredient in monogastric animal feed, particularly due to its essential amino acid profile (Methionine: 0.7% to 0.9%; Valine: 2.3% to 2.8%; Lysine: 2.3% to 2.6%; Arginine: 1.8% to 2.0%), crude protein content ( $\leq 53\%$ ), crude fat content ( $\leq 58\%$ ), and calcium content ( $\leq 7\%$ ) on a DM basis (Ewald et al., 2020; Ruhnke et al., 2018; Spranghers et al., 2017). Additionally, the rearing environment of BSFL can be controlled (e.g., rearing substrate, temperature), which facilitates production with relatively low nutrient variability, amino acid, and fatty acid profile (Ewald et al., 2020; Spranghers et al., 2017). Beyond the nutritional potential in diets, BSFL may also offer health benefits to broilers. Some of the potential health benefits come from lauric acid (C12:0), which constitutes up to 64% of the total saturated fatty acid composition of the BSFL. Fortuoso et al. (2019) as well as Londok and Rompis (2019) evaluated the potential benefits of including lauric acid in broiler diets at 0.03% and 2.6% respectively and found a significant reduction in *Escherichia coli* and total bacterial counts in excreta samples, improving intestinal health as well as broiler performance. Similarly, an in vitro study reported that BSFL micro compounds can reduce the growth of a large number of harmful microorganisms, including gram-positive *Staphylococcus aureus*, methicillin resistant *S. aureus*, and gram-negative *Pseudomonas aeruginosa* (Park et al., 2014). These results suggest that the active micro compounds identified in the larvae extract acted as antimicrobial against these microorganisms (Park et al., 2014). Chitin, as part of the BSFL exoskeleton represents a significant constituent, and has also been reported to have immunomodulatory effects in mammals on the innate and adaptive immune system (Komi et al., 2018).

The commercial application of full-fat BSFL in the broiler industry requires further development. Understanding the maximum dietary inclusion rates of full-fat BSFL when fed for the life of broilers is paramount for commercial application, including impacts on broiler performance, nutrient digestibility, and immunity. Therefore, the objective of the study was to determine the optimal rate of inclusion level of the full-fat BSFL in broiler diets and to investigate the association of different inclusion levels of BSFL in broiler diet for 42 d on performance, nutrient digestibility, and immune status.

Our specific questions were: 1) Is there any relationship between BSFL inclusions in the broiler diets and the nutrient digestibility of these diets? 2) What is the relationship between full-fat BSFL inclusions in broiler diets and broiler performance parameters? 3) What is the relationship between the commercial broiler's immunity and BSFL inclusion level? We hypothesised that control diets and BSFL diets would have no disadvantage on broiler performance, nutrient digestibility would be slightly reduced by the BSFL diets and that antimicrobial properties of the BSFL would positively affect immune parameters of broilers.

## 2. Materials and methods

### 2.1. Experimental animals and diets

Samples of feed ingredients were collected and analysed in duplicates for gross energy, DM, crude protein, crude fat, and ash

content. The nutrient composition was then used to formulate 5 experimental diets for each dietary phase; starter (2 to 10 d), grower (11 to 21 d) and finisher (22 to 42 d), resulting in a total of 15 experimental diets and one pre-starter diet (fed on d 1), all meeting the Aviagen (2016) nutrient recommendations (Table 1). Five inclusion levels of full-fat BSFL were used for all dietary phase; for the starter, 0, 2.5%, 5%, 7.5% and 10%, for grower and finisher diets, 0, 5%, 10%, 15% and 20%. Similar energy levels and digestible amino acid values were targeted across the diets to compare performance between treatments directly. All experimental diets contained 0.2% titanium dioxide (TiO<sub>2</sub>) as an indigestible marker to determine nutrient digestibility.

Four hundred, one-day-old Ross 308 sexed male broilers were obtained from a commercial hatchery and placed into 40 cages with 0.44 m<sup>2</sup> floor area each. The broilers were weighed (SB32001 DeltaRange Balance, Mettler Toledo, Painesville, Ohio, USA) and allocated to each cage in such a way that the average body weight of the broilers in each cage was comparable. Cages were then randomly allocated to one of the 5 different treatment groups allowing for ten broilers per cage and 8 cage replicates per treatment. The University of New England Animal Ethics Committee approved this research (AEC18-084).

### 2.2. Black soldier fly larvae composition

The BSFL was reared and supplied by a commercial company (Karma<sup>3</sup> Pty Ltd, Melbourne, VIC, Australia) in 4 batches. All 4 BSFL batches used for this experiment were produced under the same conditions: fed the same conventional chicken feed as a rearing substrate, harvested on the 12th day of the larvae development, and dried at 80 °C. Before being included in the diet formulations, each batch of BSFL was chemically analysed to quantify values of gross energy, dry matter (DM), crude ash, crude fat, crude protein, and minerals (Table 2). The BSFL samples were lyophilised at –50 °C and 5 Pa (FD – PILOT7 – 12 Series, Dynavac, Sydney, Australia) and then ground in a Sunbeam Multigrinder II (Botany, NSW, Australia) using a wing blade system. The ground samples were then used for further analysis. DM results were calculated using the weight difference of the fresh and dried samples according to the AOAC method number 934.01 (AOAC 2005). Crude ash was determined by incineration at 550 °C for 4 h in a muffle furnace (Carbolite Gero Limited, Hope Valley, UK) following the method described by Spranghers et al. (2017). Total nitrogen was determined by DUMAS-method (LECO TruMac CNS analyser, Leco Corporation, St. Joseph, MI, USA) and crude protein (CP) was calculated by the factor N × 6.25. Crude fat percentage was measured using the Soxhlet extractor and chloroform solvent as described by Folch et al. (1957). The amino acid composition was analysed using Ultra Performance Liquid Chromatography (UPLC) following the method described by Wheat et al. (2008). The equipment used for the UPLC method was an ACQUITY UPLC System with an UV detector (Waters Corporation, Milford, MA, USA). For amino acid analyses, a Waters AccQTag Ultra column (BEH C18, 2.1 × 100 mm; 1.7 μm) was used. The column temperature was 57 °C, with a detection set at 260 nm and a flow rate of 0.7 mL/min. The fatty acid composition of the BSFL was determined according to the method described by Clayton et al. (2012). In brief, the fatty acid profile of BSFL samples was determined as follows: approximately 200 g of BSFL samples were diluted in 5.0 mL of a chloroform-methanol solution (2:1, Vol/Vol), resulting in a solution of which the concentration ratio solvent/sample was 25:1. The resulting solution was vortexed in a centrifuge tube for 30 s and then centrifuged for 15 min at 1,500 × g to collect the supernatant. The resulting supernatant was then diluted in 0.9% NaCl (1 mL) and centrifuged again in a conical centrifuge tube for 10 min at 1,500 × g. The lower chloroform layer was collected through aspiration, then

**Table 1**  
Ingredient composition and calculated nutrient composition of treatment diets.

Item	Starter diets <sup>1</sup>					Growers diets <sup>1</sup>					Finishers diets <sup>1</sup>				
	T1	T2	T3	T4	T5	T1	T2	T3	T4	T5	T1	T2	T3	T4	T5
<b>Ingredients, %</b>															
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
Soybean meal	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
BSFL	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
Meat and bone meal	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 <sub>4</sub>	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
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 bicarbonate	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 <sub>2</sub>	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
Vitamin premix <sup>2</sup>	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
Mineral premix <sup>3</sup>	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
Phytase <sup>4</sup>	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
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
L-Lysine HCl (78.4)	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
D,L-Methionine	0.34	0.34	0.33	0.31	0.30	0.35	0.32	0.3	0.28	0.27	0.23	0.21	0.19	0.16	0.13
L-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
L-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
<b>Calculated composition, %</b>															
ME, kcal/kg	3,000	3,000	3,000	3,000	3,000	3,100	3,100	3,118	3,141	3,167	3,200	3,200	3,200	3,200	3,200
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 Methionine	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 Lysine	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 Methionine + Cysteine	0.99	0.99	0.99	0.99	0.99	0.95	0.95	0.95	0.95	0.95	0.80	0.80	0.80	0.80	0.80
d Threonine	0.99	0.99	1.00	1.01	1.03	0.95	0.98	1.00	1.01	1.03	0.92	0.94	0.96	0.99	1.01
d Arginine	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 Isoleucine	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
d Valine	1.05	1.05	1.07	1.09	1.11	0.98	0.98	0.97	1.03	1.03	0.88	0.89	0.91	0.93	0.99
Calcium	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
Available phosphorus	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

BSFL = black soldier fly larvae, d = digestible.

<sup>1</sup> Starter diets were provided to the birds from 2 to 10 d. Grower diets were provided to the birds from 11 to 21 d. Finisher diets were provided to the birds from 22 to 42 d. T1: control group, no inclusion of BSFL in the diet. T2: 2.5% BSFL in the starter period followed by 5% BSFL in the grower and finisher period. T3: 5% BSFL in the starter period followed by 10% BSFL in the grower and finisher period. T4: 7.5% BSFL in the starter period followed by 15% BSFL in the grower and finisher period. T5: 10% BSFL in the starter period followed by 20% BSFL in the grower and finisher period.

<sup>2</sup> Vitamin concentrate supplied per kilogram of diet: retinol 12,000 IU; cholecalciferol 5,000 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.

<sup>3</sup> Trace mineral concentrate supplied per kilogram of diet: Cu (sulphate) 16 mg; Fe (sulphate) 40 mg; I (iodine) 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.

<sup>4</sup> AXTRA phytase 100 g/tonne (1,000 FTU/kg).

part of this chloroform solution was evaporated. The remaining portion was methylated, according to [Lepage and Roy \(1984\)](#). Individual fatty acids methyl esters were identified using an Agilent 6890 N gas chromatograph (GC) with a flame ionisation detector (FID) (Model 6890N, Agilent, Santa Clara, CA, USA).

### 2.3. Growth performance evaluation

Broilers and feed were weighed at the beginning and end of each experimental period (2, 10, 21, and 42 d) to determine feed intake and body weight (BW). Birds were observed twice daily, mortalities recorded, and the BW of dead birds included in the feed conversion ratio (FCR). Each cage was considered as one replicate, and a total of 8 replicates were used for each dietary treatment.

$$FCR = \frac{\text{Replicate feed intake}}{\text{Replicate body weight gain (alive + dead birds)}}$$

The individual feed intake was calculated as:

$$\text{Individual feed intake} = FCR \times \frac{\text{Replicate weight gain}}{\text{Number of birds per replicate}}$$

Individual body weight gain (BWG) was calculated as the average BW at the end of the period minus the average BW at the beginning of the period.

### 2.4. Nutrient digestibility

#### 2.4.1. Sample preparation

On 21 d of the experiment, 5 broilers per cage (total of 200 broilers) were stunned, decapitated, and the ileum content per replicate pooled for nutrient digestibility following the method published by [Kluth et al. \(2005\)](#) and [Rezvani et al., 2008](#). Following the same method, on 42 d three broilers per cage (total of 120 broilers) were humanely killed, and the ileal digesta content collected and pooled for each replicate. All samples were lyophilised (FD – PILOT7 – 12 Series, Dynavac, Sydney, Australia) for 7 d. The condenser temperature and the vacuum pressure was maintained at –50 °C and 5 Pa. After drying the samples, all feed and digesta samples were ground using a Sunbeam Multigrinder II (Sunbeam, Botany, NSW, Australia). Homogenised samples of starter, grower, and finisher diets were prepared for nutrient content analysis using the same method.

**Table 2**  
Black soldier fly larvae composition<sup>1</sup>.

Parameter	Mean	SD	CV, %	Parameter	Mean	SD	CV, %	Parameter	Mean	SD	CV, %
Nutrients, %			Fat acid profile, g/kg DM			Amino acid profile, mg/g DM					
DM	94.17	0.50	0.01	SFA							
Gross energy, MJ/kg	22.65	0.32	0.01	C12:0 (Lauric Acid)	128.29	13.08	0.10	Histidine	11.12	0.22	0.02
Crude protein	40.11	1.35	0.03	C14:0 (Myristic Acid)	22.27	2.10	0.09	Serine	16.13	0.49	0.03
Crude fat	32.54	3.53	0.11	C16:0 (Palmitic Acid)	32.72	2.54	0.08	Arginine	18.42	0.79	0.04
Crude ash	14.02	0.43	0.03	C10:0	6.69	0.72	0.11	Glycine	19.94	0.62	0.03
Minerals, mg/kg				C18:0	7.55	0.35	0.05	Aspartic acid	32.74	0.80	0.02
Calcium	48,200.02	2,946.47	0.06	Total SFA	200.70	18.42	9.18	Glutamic acid	43.68	2.11	0.05
Phosphorus	8,290.59	712.10	0.09	MUFA				Threonine	15.78	0.43	0.03
Aluminium	2,255.00	496.06	0.22	C18:1n-9 (Oleic Acid)	24.65	2.01	0.08	Alanine	27.62	2.64	0.10
Copper	155.00	5.00	0.03	C18:1n-7	1.25	0.09	0.07	Proline	20.92	0.49	0.02
Iron	2,697.50	248.03	0.09	C18:1n-7t	0.51	0.21	0.41	Lysine	22.57	0.66	0.03
Potassium	13,283.20	950.44	0.07	C14:1n-5	0.32	0.03	0.10	Tyrosine	21.01	0.61	0.03
Magnesium	3,933.49	183.35	0.05	C17:1n-7	0.28	0.14	0.49	Methionine	7.09	0.29	0.04
Manganese	3,562.50	181.71	0.05	Total MUFA	28.05	1.99	7.11	Valine	23.16	0.56	0.02
Sodium	9,435.00	1173.85	0.12	PUFA				Isoleucine	17.23	0.50	0.03
Sulphur	3,407.55	123.97	0.04	C18:4n-3	0.32	0.08	0.25	Leucine	27.27	0.76	0.03
Zinc	1,365.00	18.03	0.01	C20:5n-3	0.11	0.02	0.22	Phenylalanine	16.97	0.53	0.03
				C18:2n-6 (Linoleic Acid)	20.22	1.67	0.08	Total	341.66	8.18	0.02
				C18:3n-3	2.35	0.21	0.09				
				Total PUFA	23.46	1.72	7.32				

SD = standard deviation; CV = coefficient of variation; DM = dry matter; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

<sup>1</sup> Not all fatty acids are shown on the table, only the ones that are most prevalent, and therefore, values of total SFA, MUFA and PUFA are not the sum of the values presented in the table.

### 2.4.2. Analysis of nutrient content

For nutrient digestibility analysis, crude protein, crude fat, DM, crude ash, and mineral concentrations of starter, grower and finisher diets as well as from lyophilised digesta were determined. Crude protein, crude fat and DM analysis were performed as per the methods described in section 2.2. Crude ash of digesta was determined following the AOAC Official method number 942.05 (AOAC, 2005). For mineral determination, ash samples were used to quantify calcium and phosphorus using the ultrawave microwave digestion system (Milestone Ultra WAVE, Milestone Srl, Sorisole, Italy). In short, roughly 0.2 g of homogenised ash sample was digested in 4 mL of concentrated nitric acid using the single reactor chamber of the microwave in a temperature range of 110 to 240 °C and a pressure of up to 110 bar. The digested samples were transferred to a 25 mL container with deionized water. The analysis for calcium and phosphorus was carried out using an inductively coupled plasma optical emission spectrometer (ICP-OES; Agilent Australia, Victoria, Australia) at wavelengths 422.673 nm for calcium and 213.618 nm for phosphorus.

### 2.4.3. Apparent ileal digestibility

To quantify the indigestible TiO<sub>2</sub> marker in the feed and the digesta, approximately 0.1 g of dried and ground ileum content and approximately 0.2 g ground feed was accurately weighed, recorded, and ashed in a muffle furnace (Carbolite Gero Limited, Hope Valley, UK) at 580 °C for 13 h. Then, the spectrophotometry method was followed as described by Short et al. (1996).

After quantifying the TiO<sub>2</sub> content in the experimental feed and ileum samples, the apparent ileal digestibility of nutrients was calculated according to the following equation:

$$AID = \left[ \left( \frac{\text{TiO}_2 \text{ feed}}{\text{TiO}_2 \text{ ileum}} \right) \times \left( \frac{\text{Nutrient ileum}}{\text{Nutrient feed}} \right) \right] \times 100$$

### 2.5. Immune parameters

At 21 and 42 d, blood from the same 2 individual broilers per cage was collected. Furthermore, at 42 d the jejunum from one of

these 2 individual broilers was used to determine the intra-epithelial lymphocytes.

### 2.5.1. Haematology

Blood samples from the same individual broilers (2 broilers/cage) were collected at 2 different ages (21 and 42 d) to determine blood cells. At 21 d, whole blood from the brachial vein was collected and placed in 4 mL plastic EDTA vacutainers (BD Vacutainer, Plymouth, UK). After the blood collection at 21 d, all broilers who had their blood collected were leg banded to be then identified on the next sampling day. On 42 d, the same 2 broilers per cage were used for blood collection as well as for jejunum sampling to determine the intestinal intraepithelial lymphocyte population.

Total blood count was analysed within 2 h after collection, using a CELL-DYN 3700 analyser (Abbott Laboratories, Illinois, USA) following the procedure described by Abbott et al. (2003). Haematological parameters measured included total white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, basophils, total erythrocytes, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and platelets. Mean values obtained from the 2 broilers per cage at each time point were used for statistical analysis.

### 2.5.2. Flow cytometric analysis

The cellular mucosal immune system status of the broiler's intestinal tract, intraepithelial lymphocytes (CD3+ T lymphocytes, CD3+CD8+ cells, CD3+CD4+ cells, and CD3+CD4+CD8 cells) were measured. At post mortem, intraepithelial T lymphocytes were estimated from isolated jejunum of 40 birds (1 bird per cage) at 42 d of age according to the method defined by Röhe (2014). The intestinal cell suspensions were stained with either a cocktail of T lymphocyte CD marker antibodies (CD3-AF647, CD4-FITC and CD8-PE; Southern Biotech, Birmingham, AL, USA) or a cocktail of isotype control antibodies (IgG1-PE, IgG1-FITC, and IgG1-AF647; Southern Biotech, Birmingham, AL, USA) for 30 min on ice in the dark according to the manufacturer's instructions.



Flow cytometric data were acquired on an Amnis FlowSight imaging flow cytometer (Millipore, Burlington, Massachusetts, USA). CD4-FITC and CD8-PE were detected using the 488 nm laser, and the CD3-AF647 was detected using the 633 nm laser. Signals from the isotype antibody cocktail were subtracted 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 of cytotoxic T lymphocytes (CD3+CD8+), T helper lymphocyte (CD3+CD4+) and double-stained T lymphocytes (CD4+CD8+).

## 2.6. Statistical analysis

The effect of BSFL dietary inclusion was analysed using a polynomial regression model at different degrees (1, 2 and 3) in JMP Statistics software (v14 IBM SAS Institute Inc., Cary, NC, USA) with body weight, body weight gain, ileal nutrient digestibility and also total blood count as response variables. The relationships that presented either a significant *P*-value or the highest  $R^2$ , indicating the best relationship representation between the variables were presented in the figures with their respective best polynomial fit curves, equations and *P*-values for each different dietary phase (10, 21, and 42 d) as well as the entire experimental period (2 to 42 d). Blood cell count results were tested for normality using the Kolmogorov–Smirnov test (SPSS software v22, IBM, Amork, NY, USA). Total blood count results were then log-transformed to normalise data before the polynomial regression analysis was conducted *P*-values less than 0.05 were considered significant.

All graphs, except the graph for flow cytometry data, were built in the open source software RStudio (v1.1.453, RStudio, Inc) using the 'ggplot2' package (Wickham, 2016). The flow cytometric data were analysed performing univariate linear regression in SPSS (v22, IBM, Amork, NY, USA), and graphs were built using IDEAS software version 6.0.340.0 (Millipore, Burlington, Massachusetts, USA).

## 3. Results

### 3.1. Broiler performance

During the starter period (2 to 10 d), parameters such as BW (Fig. 1A), feed intake (Fig. 2A) and BWG (Fig. 3A) were not associated with BSFL inclusion in broiler diets at any degree. FCR had a cubic relationship with BSFL inclusion levels during the starter period (Fig. 4A;  $P = 0.043$ ,  $R^2 = 0.2$ ).

Furthermore, during the grower period (10 to 21 d), there was no relationship between BSFL level and performance parameters and, except for individual BW and BWG, where there was a positive linear association ( $P = 0.004$ ;  $R^2 = 0.2$ ) between BSFL levels and BW of broilers as well as a quadratic association between these factors at 21 d when grower diets (Table 1) were offered (Fig. 1B;  $P = 0.01$ ;  $R^2 = 0.22$ ). Body weight gain (BWG) of broilers was also linearly increased with BSFL inclusion in the diets (Fig. 3B;  $P = 0.017$ ;  $R^2 = 0.141$ ).

Within the finisher phase (21 to 42 d), individual BW positively correlated with greater dietary inclusion of BSFL in a positive linear relationship with body weight at 42 d ( $P = 0.024$ ;  $R^2 = 0.128$ ). The inclusion of BSFL in broiler diets had a quadratic relationship with BW (Fig. 1C;  $P = 0.026$ ;  $R^2 = 0.178$ ), indicating that the broiler's BW had some draw backs at lower BSFL levels. FCR and BSFL diet inclusion also demonstrated a negative relationship at 42 d with a decrease from 1.5 to 1.4 when comparing the control group to the 20% BSFL inclusion (Fig. 4C; quadratic effect;  $P = 0.034$ ,  $R^2 = 0.166$ ).

When the broiler performance parameters of the entire experimental period (2 to 42 d) were calculated, there was no relationship between BSFL diets and the feed intake (Fig. 2D), but BWG linearly increased when 20% of BSFL was included in the diets compared to the control diets ( $P = 0.022$ ;  $R^2 = 0.134$ ). Body weight gain calculation for the entire period also had a quadratic relationship with BSFL levels in the diet (Fig. 3D;  $P = 0.024$ ;  $R^2 = 0.187$ ). The FCR calculation from 2 to 42 d linearly reduced by 10% (from 1.54 to 1.38 g/g) when up to 20% BSFL was added in the diets ( $P = 0.003$ ;  $R^2 = 0.211$ ). The relationship between FCR and BSFL levels was also significant for both quadratic (Fig. 4D;  $P = 0.001$ ;  $R^2 = 0.34$ ) and cubic responses ( $P = 0.002$ ;  $R^2 = 0.34$ ).

### 3.2. Nutrient digestibility

At 21 d, there was no effect of BSFL inclusion on DM, crude protein, crude ash, and calcium, or phosphorus digestibility (Figs. 5–7, respectively), however, there was an increase in crude fat digestibility (linear response; Fig. 6A;  $P = 0.002$ ,  $R^2 = 0.337$ ). Furthermore, no effect was observed on crude protein, crude fat, crude ash, or calcium digestibility at 42 d, but a negative linear relationship was observed for DM digestibility (Fig. 5B;  $P = 0.018$ ;  $R^2 = 0.138$ ). Additionally, the digestibility of phosphorus diminished with increasing levels of BSFL in the diet (Fig. 7B; quadratic response;  $P = 0.0002$ ;  $R^2 = 0.452$ ).

### 3.3. Immune parameters

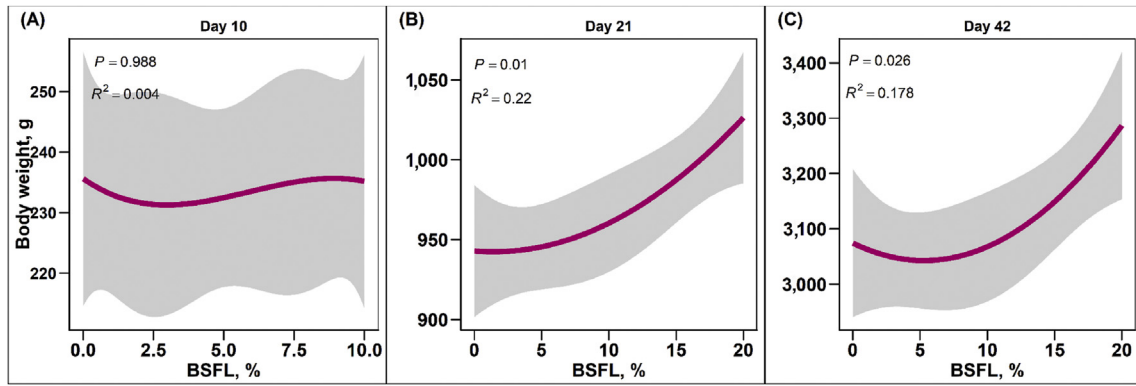
#### 3.3.1. Haematology

When measured at 21 d (Fig. 8A), the inclusion of BSFL in the broiler diets decreased peripheral white blood cells by 35.9%, from  $50.2 \times 10^6/\text{mL}$  (control diet) to  $32.2 \times 10^6/\text{mL}$  (20% BSFL inclusion) ( $P = 0.001$ ,  $R^2 = 0.34$ ). Peripheral blood lymphocytes linearly decreased by 50% from  $39.4 \times 10^6/\text{mL}$  to  $20.7 \times 10^6/\text{mL}$  (Fig. 8C;  $P < 0.001$ ;  $R^2 = 0.40$ ).

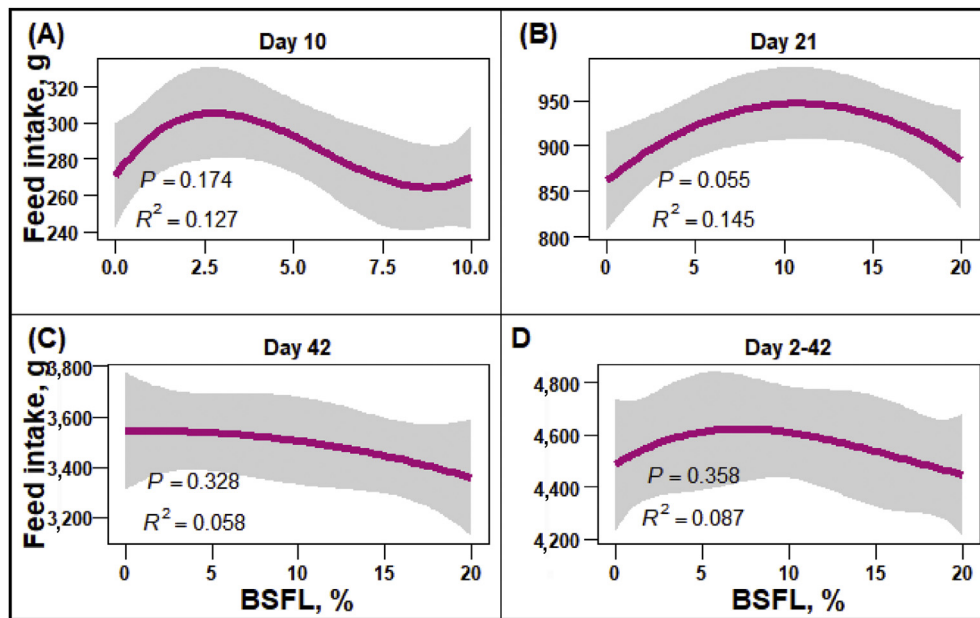
At 42 d, there was a negative linear relationship between the number of white blood cells and the dietary BSFL inclusion levels (Fig. 8B; linear response;  $P = 0.027$ ,  $R^2 = 0.062$ ).

#### 3.3.2. Intraepithelial lymphocytes

With increasing BSFL levels in the diets, the number of jejunal intraepithelial CD3+ T lymphocytes decreased ( $P = 0.025$ ; Fig. 9). There was a 4-fold decrease in CD3+ T lymphocytes that were observed between hens fed the control diet (no BSFL) and the 20% BSFL diet. As expected, CD3+CD8 cytotoxic T lymphocytes were the major (approximately 58%) intraepithelial intestinal CD3+ T lymphocyte subpopulation present in broilers fed the control diet followed by CD3+CD4+CD8+ subtypes (13%) and then CD3+CD4+ T helper lymphocytes (10%). There was a decrease in the subpopulations of CD3+CD8+ cytotoxic intestinal T lymphocytes with increasing BSFL levels, which accounted for 20.5% of the observed variance ( $P = 0.016$ ). The broilers fed a 20% BSFL supplemented diet had a 9.7-fold decrease in intestinal intraepithelial CD3+CD8+ lymphocytes compared to broilers that were fed with a control diet (Fig. 9D). Similarly, there was a 5.7-fold decrease ( $P = 0.004$ ; 28.3% of the variance) in CD3+CD4+CD8+ T lymphocytes in the intestines of broilers fed with a 20% larval diet compared to broilers fed the control diet (Fig. 9F). The data suggest that the reduction of intestinal cytotoxic T lymphocyte and CD4+CD8+ double-stained T lymphocyte populations are associated with BSFL inclusion in broilers diets. In contrast, there was no impact on the intestinal T helper (CD3+CD4+) intraepithelial lymphocyte populations by feeding broilers BSFL.



**Fig. 1.** Final body weight of broilers (Ross 308) fed diets with up to 20% black soldier fly (BSFL) at the end of each phase (starter at 10 d [A], grower at 21 d [B] and finisher at 42 d [C]). All figures have polynomial fit curves that best describe the relationship between body weight and BSFL levels. (A) Body weight (10 d) =  $227.51 + 0.99 \times \text{BSFL} (\%) + 0.12 \times [\text{BSFL} (\%) - 5]^2 - 0.04 \times [\text{BSFL} (\%) - 5]^3$ ; (B) Body weight (21 d) =  $918.47 + 8.36 \times \text{BSFL} (\%) + 0.971 \times [\text{BSFL} (\%) - 5]^2$ ; (C) Body weight (42 d) =  $2,961.48 + 10.63 \times \text{BSFL} (\%) + 1.13 \times [\text{BSFL} (\%) - 10]^2$ .



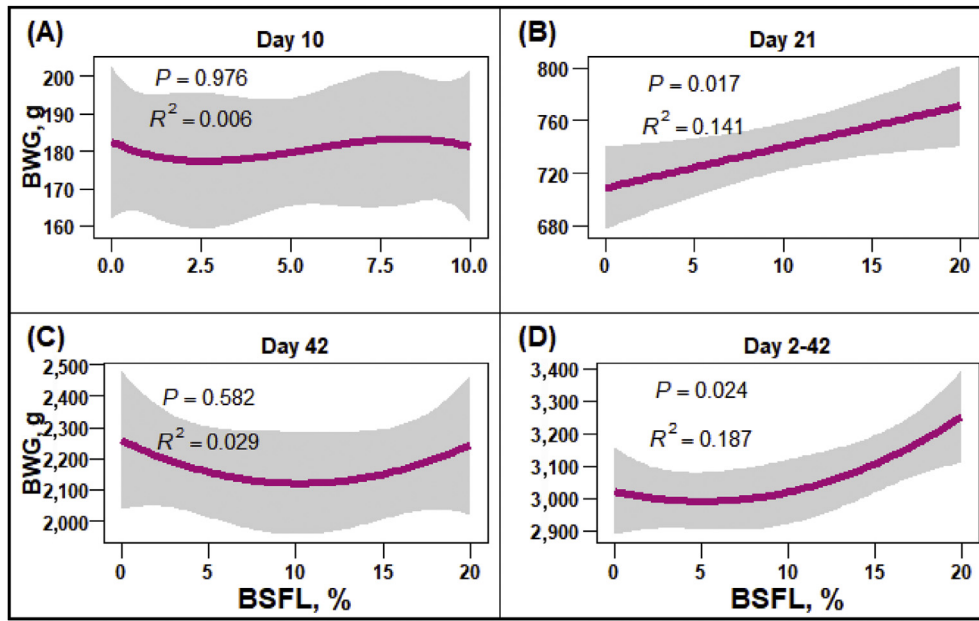
**Fig. 2.** Feed intake of broilers (Ross 308) fed diets with up to 20% black soldier fly larvae (BSFL) at the end of each phase (starter at 10 d [A], grower at 21 d [B] and finisher at 42 d [C]) as well as the total feed intake (from 2 to 42 d [D]). All figures have polynomial fit curves that best describe the relationship between feed intake and BSFL levels. (A) Feed intake (10 d) =  $340.46 - 9.52 \times \text{BSFL} (\%) - 0.89 \times [\text{BSFL} (\%) - 5]^2 + 0.38 \times [\text{BSFL} (\%) - 5]^3$ ; (B) Feed intake (21 d) =  $934.49 + 2.40 \times \text{BSFL} (\%) - 2.94 \times [\text{BSFL} (\%) - 5]^2$ ; (C) Feed intake (42 d) =  $3,754.41 - 15.051 \times \text{BSFL} (\%) - 1.35 \times 10^{-10}$ ; (D) Feed intake (2 d – 42 d) =  $4,881.00 - 14.71 \times \text{BSFL} - 1.76 \times (\text{BSFL} (\%) - 9.74)^2 + 0.04 \times (\text{BSFL} (\%) - 9.74)^3$ .

## 4. Discussion

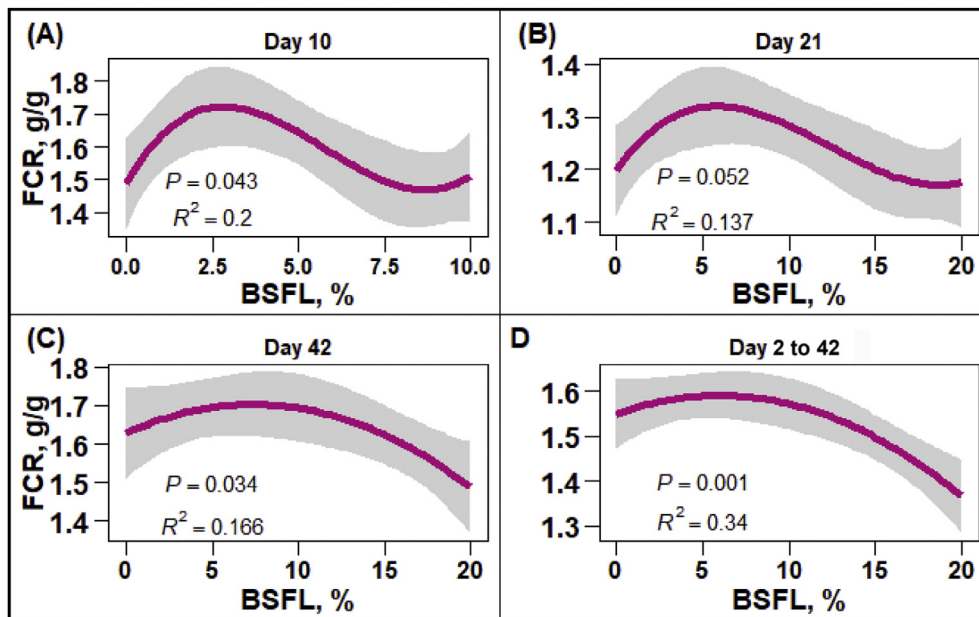
### 4.1. Growth performance

Overall, FCR of broilers improved with increasing BSFL levels in the diets. This was mostly due to the effect of the BSFL inclusion levels on growth resulting in a significant increase in grower and finisher BW. Similar effects were observed by [Bovera et al. \(2016\)](#). They also reported improved FCR from 4.1 to 3.6 in Shaver brown broiler chickens fed diets containing *Tenebrio molitor* meal at 23.7% for 32 d (from 30 d until 62 d of age). Similarly, other research studies have also observed a positive performance impact of dietary inclusion of insect meal such as higher BW, BWG or lower FCR in at least one phase during the experiment in broilers ([Dabbou et al., 2018](#); [Gariglio et al., 2019](#); [Khan et al., 2018](#); [Loponte et al., 2017](#)).

In contrast, [Onsongo et al. \(2018\)](#) did not find any effect of insect diets on performance parameters (feed intake, FCR, or BWG) in broilers when being fed with BSFL at different inclusion levels up to 15%. The improvement of performance in the broilers fed BSFL meals in this study may be explained by the relatively high levels of lauric acid in the BSFL (128.3 g/kg DM in this study), resulting in up to 29.4 g lauric acid/kg diet. [Fortuoso et al. \(2019\)](#), included up to 300 mg of lauric acid per kilogram of feed in broiler diets and reported an increase of more than 11% in BWG and a 6% reduction in FCR, suggesting that lauric acid demonstrated strong antimicrobial effect and growth promoter ability with no toxicity. Additionally, 53 genes encoding apparent antimicrobial peptides present in the BSFL have proven antibiotic activities when being used in vitro at a minimal inhibitory concentration of 25 mg/mL with clinical research not yet being conducted ([Park et al., 2014](#); [Vogel et al., 2018](#)). It might also be possible that the chitin content of the BSFL



**Fig. 3.** Body weight gain (BWG) of broilers (Ross 308) fed diets with up to 20% black soldier fly larvae (BSFL) at the end of each phase (starter at 10 d [A], grower at 21 d [B] and finisher at 42 d [C]) as well as the total feed intake (from 2 to 42 d [D]). All figures have linear and polynomial fit curves that best describe the relationship between body weight gain and BSFL levels. (A) Body weight gain (10 d) =  $171.80 + 1.57 \times \text{BSFL} (\%) + 0.09 \times [\text{BSFL} (\%) - 5]^2 - 0.07 \times [\text{BSFL} (\%) - 5]^3$ ; (B) Body weight gain (21 d) =  $708.68 + 6.25 \times \text{BSFL} (\%)$ ; (C) Body weight gain (42 d) =  $2,128.56 - 0.75 \times \text{BSFL} (\%) + 1.30 \times [\text{BSFL} (\%) - 10]^2$ ; (D) Body weight gain (2 - 42 d) =  $2,909.73 + 10.86 \times \text{BSFL} (\%) + 1.18 \times [\text{BSFL} (\%) - 9.74]^2$ .

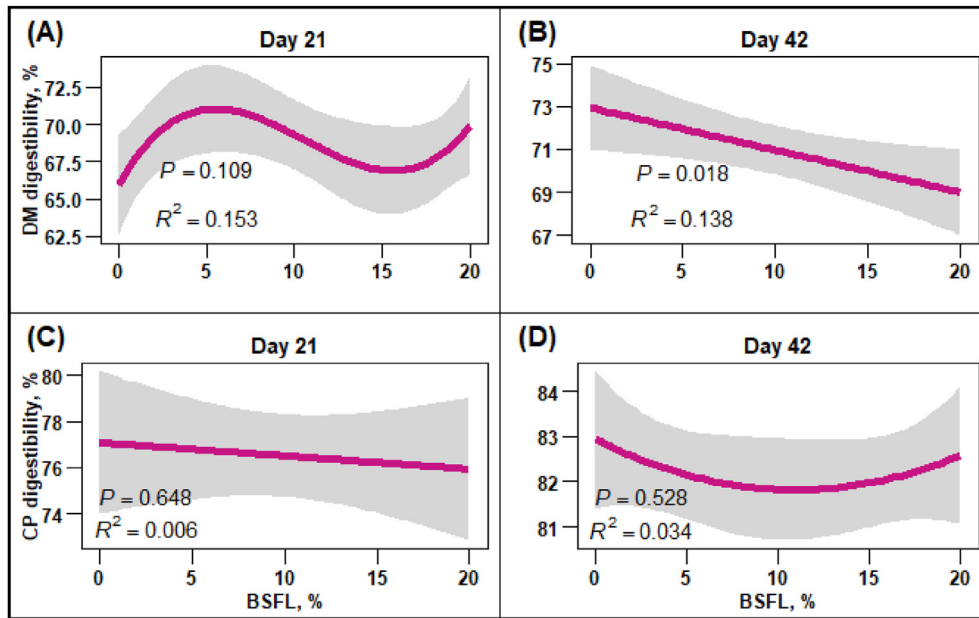


**Fig. 4.** Feed conversion ratio (FCR) of broilers (Ross 308) fed diets with up to 20% black soldier fly larvae (BSFL) at the end of each phase (starter at 10 d [A], grower at 21 d [B] and finisher at 42 d [C]) as well as the total feed intake (from 2 to 42 d [D]). All figures have polynomial fit curves that best describe the relationship between FCR and BSFL levels. (A) FCR (10 d) =  $1.95 - 0.06 \times \text{BSFL} (\%) - 0.01 \times [\text{BSFL} (\%) - 5]^2 + 0.002 \times [\text{BSFL} (\%) - 5]^3$ ; (B) FCR (21 d) =  $1.44 - 0.03 \times \text{BSFL} (\%) - 0.004 \times [\text{BSFL} (\%) - 5]^2 + 0.001 \times [\text{BSFL} (\%) - 5]^3$ ; (C) FCR (42 d) =  $1.76 - 0.007 \times \text{BSFL} (\%) - 0.001 \times \text{BSFL} (\%) - 10]^2$ ; (D) FCR (2 - 42 d) =  $1.65 - 0.008 \times \text{BSFL} (\%) - 0.001 \times [\text{BSFL} (\%) - 10.13]^2$ .

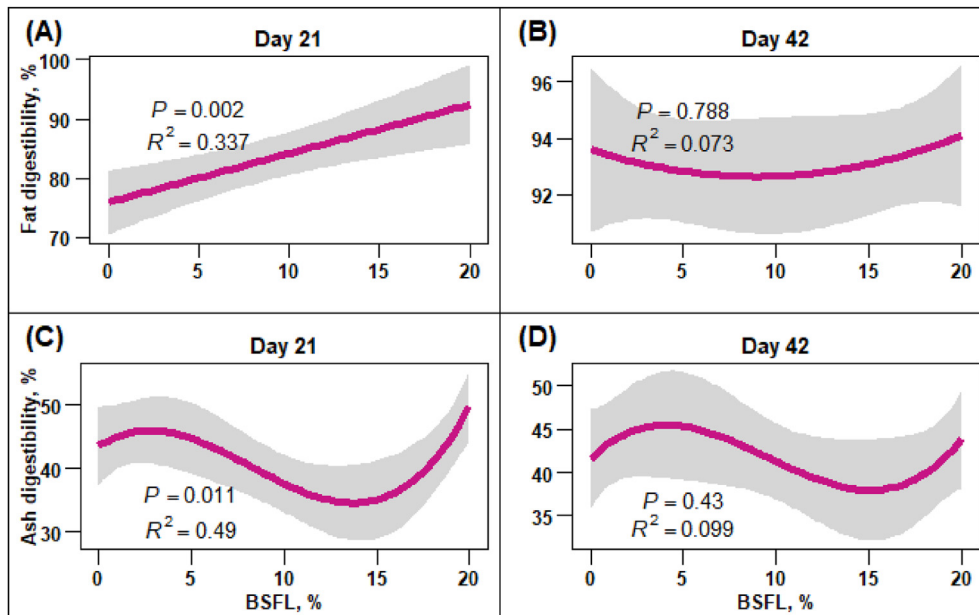
(4.62% DM) acted as a prebiotic in the intestine of the broilers as described, resulting in a decreased DM digestibility and improved FCR (Bovera et al., 2016). Our findings of a quadratic impact of the BSFL on broiler performance may indicate a similar effect and would need further investigation. Equivocal results have been observed from investigators using up to 15% of BSFL in their diets, but the larvae composition varies greatly between studies due to the use of full or partially defatted larvae and its associated changes

on nutrients but also comparing treatment groups that were not formulated based on the same concept (Dabbou et al., 2018; Onsongo et al., 2018).

Based on the fact that the diets used in the current experiment were of very similar caloric density and balanced in their amino acid composition, broilers fed up to 20% full-fat BSFL in a balanced diet had greater performance compared to those fed a control diet, having the optimal inclusion level between 15% and 20%. However,



**Fig. 5.** Dry matter (DM; [A] 21 d; [B] 42 d) and crude protein (CP; [C] 21 d; [D] 42 d) digestibility of broilers (Ross 308) fed diets with up to 20% black soldier fly larvae (BSFL) at 21 and 42 d. (A) and (B) have polynomial and linear fit curves that best describe the relationship between DM digestibility and BSFL levels. (C) and (D) have linear and polynomial fit curves that best describe the relationship between CP digestibility and BSFL levels. (A) DM digestibility (21 d) =  $75.40 - 0.61 \times \text{BSFL} (\%) - 0.014 \times [\text{BSFL} (\%) - 10]^2 + 0.008 \times [\text{BSFL} (\%) - 10]^3$ ; (B) DM digestibility (42 d) =  $72.94 - 0.20 \times \text{BSFL} (\%)$ ; (C) CP digestibility (21 d) =  $77.09 - 0.06 \times \text{BSFL} (\%)$ ; (D) CP digestibility (42 d) =  $82.0 - 0.02 \times \text{BSFL} (\%) + 0.01 \times [\text{BSFL} (\%) - 10]^2$ .



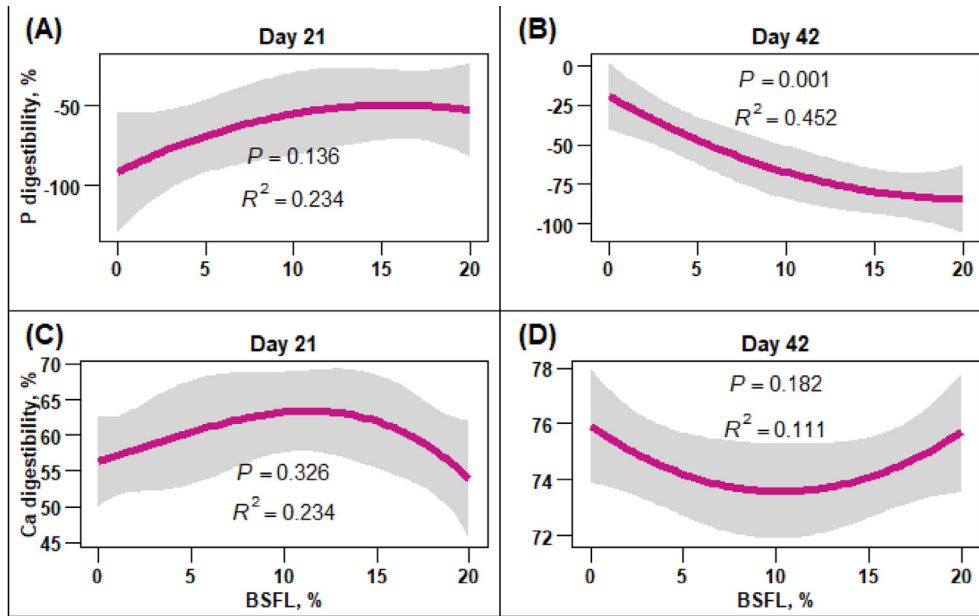
**Fig. 6.** Crude fat ([A] 21 d; [B] 42 d) and crude ash ([C] 21 d; [D] 42 d) digestibility of broilers (Ross 308) fed diets with up to 20% black soldier fly larvae (BSFL) at 21 and 42 d. (A) and (B) have linear and polynomial fit curves that best describe the relationship between crude fat digestibility and BSFL levels at 21 and 42 d. (C) and (D) have polynomial fit curves that best describe the relationship between ash digestibility and BSFL levels. (A) Crude fat digestibility (21 d) =  $75.86 + 0.83 \times \text{BSFL} (\%)$ ; (B) Crude fat digestibility (42 d) =  $89.5 + 0.07 \times \text{BSFL} (\%) + 0.04 \times [\text{BSFL} (\%) - 10.69]^2$ ; (C) Ash digestibility (21 d) =  $51.11 - 1.35 \times \text{BSFL} (\%) + 0.1 \times [\text{BSFL} (\%) - 10.25]^2 + 0.02 \times [\text{BSFL} (\%) - 10.25]^3$ ; (D) Ash digestibility (42 d) =  $51.47 - 1.02 \times \text{BSFL} (\%) + 0.02 \times [\text{BSFL} (\%) - 10.17]^2 + 0.01 \times [\text{BSFL} (\%) - 10.17]^3$ .

especially the grower diets varied up to 4% in their crude protein and metabolic energy, which may have confounded the observed results. Further research to determine the impact of this variation is, therefore warranted.

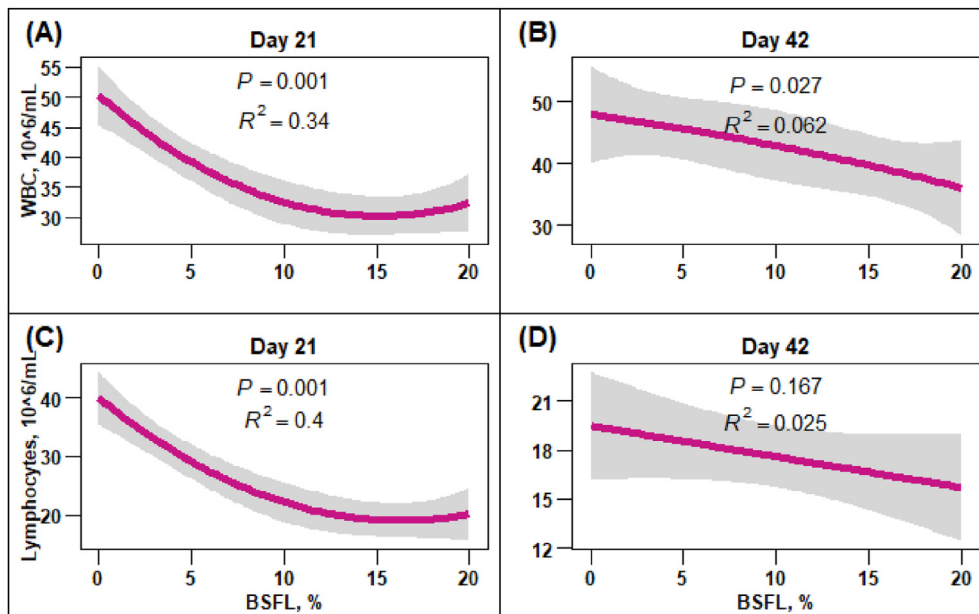
There are current constraints to include BSFL in the poultry industry on a large scale. Depending on the country, the cost of the BSFL may not be competitive in comparison to conventional protein

meals and the scale required to produce BSFL at an attractive price involves drastic changes to current BSFL production systems. However, as previously stated, BSFL is an attractive feed ingredient for animal production systems in some countries due to natural resource availability and a weak currency for protein meal importation. The suggested optimal inclusion level of 15% to 20% will require the development of BSFL production in many countries to





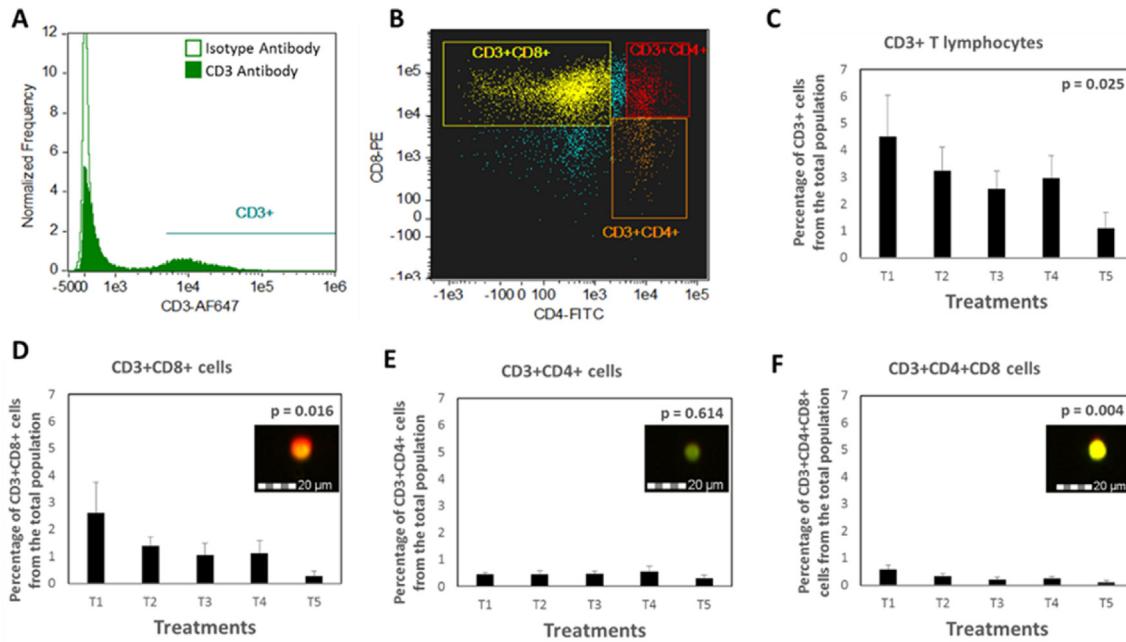
**Fig. 7.** Phosphorus (P; [A] 21 d; [B] 42 d) and calcium (Ca; [C] 21 d; [D] 42 d) digestibility of broilers (Ross 308) fed diets with up to 20% black soldier fly larvae (BSFL) at 21 and 42 d. All figures have polynomial fit curves that best describe the relationship between the nutrient digestibility and BSFL levels. (A) Ca digestibility (21 d) =  $62.76 + 0.06 \times \text{BSFL} (\%) - 0.08 \times [\text{BSFL} (\%) - 9.17]^2$ ; (B) Ca digestibility (42 d) =  $73.70 - 0.01 \times \text{BSFL} (\%) + 0.02 \times [\text{BSFL} (\%) - 10]^2$ ; (C) P digestibility (21 d) =  $87.59 + 3.30 \times \text{BSFL} (\%) - 0.23 \times [\text{BSFL} (\%) - 11.11]^2 - 0.02 \times [\text{BSFL} (\%) - 11.11]^3$ ; (D) P digestibility (42 d) =  $35.76 - 3.15 \times \text{BSFL} (\%) + 0.15 \times [\text{BSFL} (\%) - 10.32]^2$ .



**Fig. 8.** Total white blood cells (WBC; [A] 21 d; [B] 42 d) and lymphocytes count ([C] 21 d; [D] 42 d) of broilers (Ross 308) fed diets with up to 20% black soldier fly larvae (BSFL) at 21 and 42 d. (A) and (B) have polynomial and linear fit curves that best describe the relationship between white blood cell count and BSFL levels. (C) and (D) have polynomial and linear fit curves that best describe the relationship between lymphocytes and BSFL levels. (A) WBC (21 d) =  $45.16 - 0.74 \times \text{BSFL} (\%) + 0.04 \times [\text{BSFL} (\%) - 10.06]^2$ ; (B) WBC (42 d) =  $48.39 - 0.592 \times \text{BSFL} (\%)$ ; (C) Lymphocytes (21 d) =  $25.54 - 0.60 \times \text{BSFL} (\%) + 0.047 \times [\text{BSFL} (\%) - 10.06]^2$ ; (D) Lymphocytes (42 d) =  $19.47 - 0.19 \times \text{BSFL} (\%)$ .

improve cost and increase volume before it is considered in many poultry diets. During diet formulation, we encountered restrictions in increasing the BSFL due to the high nutrient density and especially the high fat content (32.5%; Table 2) of the full-fat BSFL. High-fat levels in the BSFL became a restriction for 2 reasons: (1) pre-established energy requirements in each dietary phase of the

broilers were respected and not exceeded; (2) the fatty acid profile including the linoleic acid concentration of the BSFL is variable and may require the addition of a second linoleic acid source such as cottonseed oil. Using full or partially extracted BSFL would facilitate diet formulations but may increase feed costs due to the additional treatment of this feed ingredient.



**Fig. 9.** Different types of intraepithelial lymphocytes response of broilers (Ross 308) fed diets with up to 20% black soldier fly larvae (BSFL). (A) Represents a histogram overlay of intact CD3+ cells intestinal T lymphocytes; (B) Shows the gating strategy of the intact CD3+ lymphocyte population defining the CD3+CD4+, CD3+CD8+ and CD3+CD4+CD8+ subpopulations of CD3+ intestinal lymphocytes; (C) represents the change in CD3+ intestinal T lymphocyte populations associated with BSFL proportions in their diet ( $n = 8$ ); (D) shows the change in CD3+CD8+ intestinal T cytotoxic lymphocyte populations associated with BSFL proportions in their diet ( $n = 8$ ); (E) refers to the change in CD3+CD4+ intestinal T helper lymphocyte populations associated with BSFL proportions in their diet ( $n = 8$ ); (F) shows the change in CD3+CD4+CD8+ intestinal T lymphocyte populations associated with BSFL proportions in their diet ( $n = 8$ ).

#### 4.2. Nutrient digestibility

DM digestibility significantly decreased at 42 d, whereas crude protein (CP) digestibility was not affected by the diets at any phase. Similarly, Bovera et al. (2016) and Cutrignelli et al. (2018), reported a reduction not only in DM digestibility but also in CP digestibility when high levels of defatted BSFL (17%) and *T. molitor* (29.7%) were included in laying hen and broiler diets, respectively. In contrast, Benzertiha et al. (2019) and Gariglio et al. (2019) reported no effect on nutrient digestibility in broilers or ducks when lower levels (up to 9%) of insect meal (*T. molitor* meal or BSFL) were added to the diets. The reduction in DM digestibility with the increased levels of chitin in the diets was expected: for example, Khempaka et al. (2006) included 0, 4%, 8%, 12% and 16% of shrimp meal (30% chitin) in broiler diets and observed a decrease in DM digestibility only when 8%, 12% and 16% of shrimp meal were included in the diet. Assuming that 4% of shrimp meal with 30% of chitin had previously no effect, one would not expect the use of 20% BSFL with 4% to 9% of chitin to affect DM digestibility in broiler diets.

#### 4.3. Immune parameters

Peripheral white blood cells in this study were significantly decreased with increased BSFL inclusion rates when evaluated at 21 and 42 d. In contrast, blood lymphocytes were significantly reduced at 21 d, but not at 42 d. There is a lack of studies reporting total blood cell count in broilers when the BSFL was included in their diets. However, Wallace et al. (2017) added BSFL (up to 14.3%) in guinea fowl diets. They reported no significant difference in white blood cells and an increase of a maximum of 58% in blood lymphocytes when the BSFL levels increased in the diets. The difference in white blood cells and peripheral blood lymphocytes response to higher levels of BSFL from this study to Wallace et al. (2017) may be

due to differences in poultry species, larvae composition, and/or the concentration of bioactive compounds.

The inclusion of BSFL in the broiler diets reduced not only peripheral blood lymphocytes but the percentage of intraepithelial cytotoxic T cells; CD3+CD8+ lymphocytes. Cytotoxic T lymphocytes kill virally infected cells, whereas intraepithelial CD3+CD4+ T-helper lymphocytes regulate the adaptive immune responses (Austyn and Wood 1993). Huo et al. (2019) reported that different lipid sources could modulate the innate immune system of broilers by reducing CD4+ T lymphocytes as well as changing the ratio between CD4+ T-helper and CD8+ cytotoxic T lymphocytes, which may indicate that the BSFL fatty acid profile affected the intraepithelial lymphocytes in this study. Furthermore, Fortuoso et al. (2019) and Londok and Rompis (2019) demonstrated the antimicrobial properties of the most prevalent fatty acid in the BSFL, the lauric acid. In addition to high levels of lauric acid (128.3 g/kg of BSFL DM in this study), BSFL can contain antimicrobial peptides as well as up to 9% of chitin (De Souza-Vilela et al., 2019). The BSFL antimicrobial peptides could be isolated in an aqueous extract showing broad antibacterial activity (Mylonakis et al., 2016; Park et al., 2014).

In vitro and in vivo studies feeding chitin and chitosan (chitin precursor) have shown their antimicrobial activities in mice (Koide 1998; Lee et al., 2009; Sayari et al., 2016). Interestingly, Ekmekci et al. (2017) also reported a reduction in CD8+ cytotoxic T lymphocytes in mice subjected to antibiotic treatment. Thus, the BSFL fatty acid profile and the expression of antimicrobial peptides, as well as chitin, may provide effective an antimicrobial barrier thereby reducing the need for high intraepithelial cytotoxic T lymphocytes in the gut of chickens. Interestingly, Schiavone et al. (2017) replaced up to 100% of soybean oil in broiler diets (up to 6.9% BSFL fat included in the diets) until 35 d of age and reported no impact on blood parameters including red and white blood cells. Here, by adding 20% of BSFL (32.5% fat) in the diets we added the

equivalent of 6.4% of BSFL fat in the broiler diets until 42 d and positively impacted white blood cells, especially lymphocytes. The comparison of these 2 studies – Schiavone et al. (2017) and ours – indicate that either the fatty acid profile of the BSFL is not the reason of the white blood cell or lymphocytes reduction, or that these reductions were a consequence of long-term effects of the BSFL fat in the immune system of the broilers.

To our knowledge, this is the first study to report that BSFL in broiler diets significantly decreased peripheral blood and jejunal intraepithelial lymphocytes populations in a dose-dependent manner. This decrease might have been (partially) responsible for the improved performance parameters, as the energy that otherwise would have been directed to immune functions would have been available for growth in birds fed BSFL. Further investigation of the effect of BSFL dietary inclusion on host immune responses to gastrointestinal bacterial and viral pathogens is warranted. In vitro studies have demonstrated the potential of antibiotic peptides extracted from BSFL to reduce the concentration of gram-positive bacteria such as *Bacillus subtilis*, or gram-negative bacteria such as *E. coli* and *Enterobacter aerogenes* and fungus (e.g. *Candida albicans*; Park et al., 2014). Moreover, an examination of the consequence of BSFL dietary inclusion on mucosal immune responses to avian gastrointestinal viruses such as rotaviruses, coronaviruses, enteroviruses, adenoviruses, astroviruses, and reoviruses (Guy, 1998) may also be considered. Thus, defined *in vivo* challenge models may prove useful for investigating the potential protective or detrimental impact on broiler immunity of a diet supplemented with BSFL.

## 5. Conclusions

Based on the results of this study, up to 20%, full-fat BSFL can be safely used in balanced broiler diet formulations without compromising broiler performance or health, and an inclusion level of 15% to 20% impacted immunologic parameters. The use of BSFL in broiler diets at that level improved growth performance parameters potentially due to the reduced energy demands of the immune system. Research conducted on commercial farms and in disease challenge models is highly warranted to validate the current results for economic relevance.

## Author contributions

**Jessica de Souza Vilela:** Conceptualisation, funding acquisition, project administration, formal analysis, preparation, creation and/or presentation of the published work, specifically visualization/data presentation, software, writing - original draft, writing - review & editing **Nicholas M. Andronicos:** Methodology, resources, formal analysis and writing - review & editing. **Manisha Kolakshyapati:** Methodology, writing - original draft and writing - review & editing. **Matthew Hilliar:** Methodology, resources and writing - review & editing. **Terence Z. Sibanda:** Data curation and visualization. **Nigel R. Andrew:** Conceptualization, writing - review & editing and supervision. **Robert A. Swick:** Methodology, writing - review & editing and supervision. **Stuart Wilkinson:** Conceptualization and writing - review & editing **Isabelle Ruhnke:** Conceptualization, funding acquisition, project administration, supervision, writing, review & editing.

## Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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