



RESEARCH ARTICLE

Substitution of Soybean Meal with Black Soldier Fly (*Hermetia illucens*) Larvae Meal in Broiler Diets: Comprehensive Effects on Growth, Gut Health and Physiological Resilience

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ABSTRACT

This research evaluated the potential of black soldier fly larvae meal (BSFLM) as a partial substitute for soybean meal (SBM) in broiler diets, for their health and optimum performance. To this end, a total of 360 one-day-old male Cobb500 broiler chicks were randomly allocated to 36 floor pens and fed on basal starter (0-7 days) and grower (8-21 days) diets. During the finisher phase (22-35 days), chicks were randomly assigned to six treatment groups (six replicate pens of 10 birds per treatment) in which SBM was substituted with BSFLM at 0, 10, 20, 30, 40, or 50%, respectively. Growth performance traits were determined as pen averages for each treatment group (n=6). At the end of the trial, blood samples were randomly collected from two birds per replicate pen (n=12) to determine the plasma metabolites, antioxidant status, cytokine indices and immune responses. Additionally, jejunum and ceca specimens were obtained from two birds per replicate (n=12) to assess the jejunum histomorphology and cecal microbiota. Data was analysed through one-way ANOVA and Tukey's post hoc test for comparison of mean differences. Results showed that including BSFLM up to 30% optimized feed conversion efficiency and maximized productive performance indices (P<0.05), including final BW (R²=0.724), BW gain (R²=0.729), and the European broiler index (R²=0.699). Intermediate inclusion levels (20-30%) were associated with alterations in the overall endocrine and physiological systems, characterized by higher thyroid activity (R²=0.608, P<0.001), enhanced antioxidant potential (R²=0.732, P<0.001) and lower pro-inflammatory cytokine interleukin-1 β (R²=0.631, P<0.001). Immunological parameters, including leukocyte viability, antibody titers and lymphocyte proliferation, were also maximised at 20-30% replacement (R²=0.608-0.703, P<0.001). The intestines significantly (P \le 0.001) exhibited better histomorphological features and a more balanced microbial composition in response to the 30% BSFLM treatment compared with other treatments. In conclusion, BSFLM can effectively substitute up to 30% of SBM in broiler finisher diets without adverse effects on productive performance and physiological status, offering a sustainable and effective protein alternative supplement for poultry production.

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INTRODUCTION

Soybean meal (SBM) has long constituted the primary protein source in broiler diets, but increasing global

demand and sustainability concerns have highlighted the need for alternatives. Soybean meal (SBM) has traditionally served as the main protein ingredient in broiler diets because of its high crude protein level, well-balanced

amino acid composition, and excellent digestibility (Lumsden *et al.*, 2024). However, increasing global demand, price fluctuations, supply chain instability and sustainability concerns, including environmental impacts such as deforestation, biodiversity loss and high carbon footprint, have prompted the search for alternative protein sources in broiler diets (Wangui *et al.*, 2025).

One promising alternative is using insect meals, including black soldier fly (*Hermetia illucens*) larvae, which have been of great interest as a sustainable and nutritious dietary ingredient (Belhadj Slimen *et al.*, 2023). Black soldier fly larvae meal (BSFLM) has gained attention due to its high protein content ($\approx 40\%$), essential amino acids and bioactive compounds (van Huis, 2020). These characteristics make BSFLM a compelling option for incorporation into avian diets, possibly enhancing both feed sustainability and the economic feasibility of poultry production.

Previous research reported that substituting SBM with low levels of BSFLM, typically up to 12.5-25%, can maintain or even improve broiler growth performance, feed efficiency, carcass weights and meat quality (de Souza Vilela *et al.*, 2021; Fruci *et al.*, 2023; Baderuddin *et al.*, 2024; Lee *et al.*, 2025). It was also reported that partial BSFLM substitution improved apparent ileal amino acid digestibility and metabolizable energy (Schiavone *et al.*, 2017). In addition, BSFLM contains bioactive compounds, such as chitin, lauric acid and antimicrobial peptides, that can enhance immune organ weight, increase plasma antioxidant enzyme activities and improve beneficial cecal microbiota, intestinal mucin dynamics and gut health parameters (Fruci *et al.*, 2023; Lau *et al.*, 2024; Saini *et al.*, 2026). In contrast, several studies reported that high inclusion levels of BSFLM (typically $\geq 50\%$) may impair gut health and growth performance, indicating that the effects of BSFLM are markedly dependent on the level of dietary incorporation (Velten *et al.*, 2018; Cheng *et al.*, 2023; Lau *et al.*, 2024). Increasing BSFLM also reduced nutrient digestibility and meat quality features (Baderuddin *et al.*, 2024; Lee *et al.*, 2025).

Although numerous studies have investigated the use of BSFLM in broiler nutrition, most previous research has primarily focused on growth performance, carcass traits, or selected physiological parameters in isolation (Facey *et al.*, 2023; Fruci *et al.*, 2023; Raju *et al.*, 2024; Lee *et al.*, 2025). The reported optimal inclusion levels vary considerably (typically 10-25%), largely due to disparities in larval processing methods (full fat vs. defatted), rearing substrates, diet formulation strategies and experimental conditions. This variability has resulted in inconsistent conclusions and poorly harmonized recommendations for practical inclusion rates. More importantly, the physiological mechanisms underlying BSFLM-mediated responses, including endocrine regulation, redox balance, inflammatory modulation, immune competence and gut structural-microbial interactions, remain insufficiently characterized in broiler chickens. While some studies have reported improvements in antioxidant status or gut microbiota at low BSFLM inclusion levels and others have highlighted potential adverse effects at high inclusion levels, we hypothesized that graded substitution of SBM with BSFLM would exert dose-dependent effects on physiological balance and productive performance of

broiler chickens with biologically optimal replacement thresholds, which need to be studied. Therefore, the present study was designed to provide an integrated evaluation of dietary BSFLM substitution for SBM by simultaneously assessing growth performance, endocrine and biochemical indicators, immunological responses, antioxidant potential, inflammatory biomarkers, intestinal histomorphology and cecal microbiota in broiler chickens, thereby clarifying key mechanistic pathways associated with graded BSFLM inclusion.

MATERIALS AND METHODS

Ethical approval: All experimental procedures were approved by the Ethical Research Council of King Faisal University, Saudi Arabia (Approval No. KFU-REC-2025-MAY-ETHICS3317).

Preparation and analysis of BSFLM: Full-fat dried BSFLM was obtained from a commercial supplier (Hindustan Protein, India), finely ground (0.5mm) and incorporated into experimental diets at the designated inclusion levels. Proximate composition and amino acid profile were determined in pooled samples following AOAC methods (Latimer Jr., 2023), while fatty acid methyl esters were analyzed using gas chromatography (Renna *et al.*, 2014). The chitin content was determined by hydrolyzing the sample with HCl, adding a methanol/hexane mixture and detecting the resulting glucosamine by chromatography (Araujo *et al.*, 2022). Chemical composition data are presented in Table 1.

Table 1: The chemical composition of the black soldier fly larvae meal (BSFLM) fed to Cobb500 broiler chickens

Items	Values
Proximate analysis	(Determined per 100g DM)
DM (%)	95.67
Ash (g)	12.50
Energy (MJ)	2.32
Protein (g)	40.13
Carbohydrate (g)	10.34
Fat (g)	29.94
Fiber (g)	4.55
Calcium (mg)	3.64
Phosphorus (mg)	0.91
Essential amino acids	(g per 100g DM)
Lysine	2.90
Methionine + Cysteine	1.18
Threonine	1.64
Arginine	2.31
Isoleucine	1.96
Leucine	3.25
Valine	2.80
Fatty acids (FA)	(g per 100g of total FA)
Lauric acid (C12:0)	44.75
Myristic acid (C14:0)	9.23
Palmitic acid (C16:0)	14.17
Stearic acid (C18:0)	2.54
Palmitoleic acid (C16:1)	3.00
Oleic acid (C18:1, c9)	10.61
Linolic acid (C18:2, n6)	11.59
alpha-linolenic acid (C18:3, n3)	1.05
Saturated FA	72.04
Monounsaturated FA	14.99
Polyunsaturated FA	12.78
Chitin content	8.55% DM

Birds and experimental protocols: A total of 360 one-day-old male Cobb500 broiler chicks were randomly

allocated to 36 floor pens (1.35×1.35m²) with 5cm of wood shaving litter in an open-system house, after ensuring comparable initial body weight across groups. The chicks were given water and mash feeds ad libitum and provided with a constant daily light schedule of 23h light and 1h darkness during the first week, after which the birds were provided with 18h light and 6h darkness till the end of trial. The brooder temperature was maintained at 32°C for the first 3 days, then gradually decreased by 2°C/week until reaching a final temperature of 22-24°C. Birds were vaccinated under veterinary supervision according to a routine health program with Newcastle disease and infectious bronchitis at 5-7 days (d) of age via drinking water, infectious bursal disease at 10-12d and a Newcastle disease booster at 18-21d. Chicks were fed on basal starter (0-7d) and grower (8-21d) diets. During the finisher phase (22-35 d), chicks were assigned to six dietary treatments with SBM replaced by BSFLM at 0, 10, 20, 30, 40, or 50% (six replicate pens of 10 birds each), respectively. Diets were formulated to meet Cobb500 nutrient requirements and were isonitrogenous and isoenergetic (Table 2 and 3). Average initial BW (IBW), final BW (FBW), BW gain (BWG), feed conversion ratio (FCR), total feed intake (TFI) and the European broiler index (EBI) were calculated for each replicate pen during the finisher period (n=6). At the end of the trial, blood samples were randomly collected from two birds in each replicate for biochemical, antioxidant, cytokine and immunological analyses (n=12), following methods described in previous studies (Alzarrah *et al.*, 2021; Al-Otaibi *et al.*, 2022). Additionally, two birds per replicate were humanely euthanized in accordance with ethical standards to obtain jejunum and ceca samples (n=12) for histomorphological examination and microbial enumeration, respectively, as described in previous work (Abbas *et al.*, 2022; Nassar *et al.*, 2023).

Table 2: Composition and nutritional analysis of the starter, grower and finisher diets administered to Cobb500 broiler chickens

Ingredients per kg as fed	Starter, d 0-7	Grower, d 8-21	Finisher, d 22-35
Soybean meal (g)	320	320	300
Wheat bran (g)	23	30	30
Corn yellow grain (g)	560	550	585
Corn gluten meal (g)	52	25	0
Vegetable oil (g)	6	35	45
Di-calcium phosphate (g)	15	15	15
Limestone (g)	10	10	10
Salt (g)	4.5	4.5	4.5
Premix ¹	5.5	5.5	5.5
DL-Methionine (g)	1.5	2.5	2.5
L-Lysine (g)	1	1	1
L-Threonine (g)	1	1	1
L-Tryptophane (g)	0.5	0.5	0.5
Nutrients calculated per kg			
Metabolizable energy (MJ)	12.1	12.7	13.0
Calcium (g)	8.0	8.0	8.0
Available phosphorus (g)	4.3	4.2	4.2
Met + Cyst (total)	9.1	8.8	8.6
Nutrients determined per kg			
Moisture (g)	95.7	98.6	100.5
Ash (g)	112.2	111.5	110.8
CP (g)	220.4	205.0	183.8
CF (g)	36.6	36.5	35.1
Total lipids (g)	29.1	57.2	67.7

¹ Contents per kg of diet: 100, 40, 100, 15, 0.35, 1, 500, 3, 9, 15, 2, 4, 20, 3, 0.02 mg of Mn, Fe, Zn, Cu, Se, Iodine, choline chloride, menadione sodium bisulfite, riboflavin, pantothenic acid, folic acid, pyridoxine, niacin, thiamine, biotin and vitamin B12, respectively. While 65, 5000 and 1000IU of DL- α tocopheryl acetate, cholecalciferol and retinyl acetate, respectively.

Table 3: Composition and nutritional analysis of the experimental meals administered to Cobb500 broiler chickens

Ingredients per kg as fed	SBM replacement with BSFLM ²				
	10%	20%	30%	40%	50%
BSFLM (g)	30	60	90	120	150
Soybean meal (g)	270	240	210	180	150
Wheat bran (g)	50	65	85	100	115
Corn yellow grain (g)	570	560	545	540	530
Corn gluten meal (g)	0	0	0	0	0
Vegetable oil (g)	40	35	30	20	15
Di-calcium phosphate (g)	15	15	15	15	15
Limestone (g)	10	10	10	10	10
Salt (g)	4.5	4.5	4.5	4.5	4.5
Premix ¹	5.5	5.5	5.5	5.5	5.5
DL-Methionine (g)	2.5	2.5	2.5	2.5	2.5
L-Lysine (g)	1	1	1	1	1
L-Threonine (g)	1	1	1	1	1
L-Tryptophane (g)	0.5	0.5	0.5	0.5	0.5
Nutrients calculated per kg					
ME (MJ)	13.1	13.2	13.3	13.4	13.5
Calcium (g)	9.0	10.0	11.1	12.1	13.1
Available phosphorus (g)	4.5	4.7	5.0	5.3	5.5
Met + Cyst (total)	8.6	8.6	8.6	8.5	8.4
Nutrients determined per kg					
Moisture (g)	104.5	99.4	96.9	102.0	104.8
Ash (g)	109.3	109.5	110.6	110.8	111.0
CP (g)	184.5	184.8	185.4	186.0	186.3
CF (g)	36.1	36.6	37.6	38.3	38.8
Total lipids (g)	71.9	76.1	80.2	79.6	83.8

¹As presented in Table 2. ²Soybean meal (SBM) replacement with black soldier fly larvae meal (BSFLM).

Biochemical assay: Plasma free-T3 (fT3) was quantified using a poultry-specific ELISA kit (MBS8807417; MyBioSource, USA). Standards and plasma samples (100 μ L) were incubated in microplate wells at 37°C, followed by successive incubation stages with a biotinylated antibody, enzyme conjugate and color reagent according to the manufacturer's instructions. Optical density (OD) was measured at 450nm using an ELISA microplate reader and fT3 concentrations were calculated from a standard curve. The assay detection range was 0.13-8ng/mL, with intra- and inter-assay CVs below 8 and 10%, respectively. Additionally, aspartate aminotransferase (AST), plasma alanine aminotransferase (ALT), uric acid and creatinine levels were determined using commercial colorimetric kits (Abcam, USA) as per manufacturer's protocols. Absorbance was spectrophotometrically recorded using a microplate reader (ELx808TM, BioTek Instruments, USA).

Redox biomarkers: The systemic antioxidant potential was evaluated by quantifying plasma redox biomarkers, including total antioxidant capacity (TAOC), total superoxide dismutase (TSOD), reduced glutathione (rGSH) and malondialdehyde (MDA), using commercial colorimetric assay kits (Elabscience Biotechnology Inc., USA). Briefly, TAOC was determined by incubating plasma with reaction reagents at 37°C and measuring absorbance at 520nm; results were calculated using the manufacturer's formula, with a detection range of 0.62-145.2U/mL and intra- and inter-assay CVs of 2.7 and 8.2%, respectively. TSOD activity was measured based on inhibition of superoxide-mediated reactions, with absorbance recorded at 550nm; the detection range was 4.7-166U/mL and intra- and inter-assay CVs were 2.8 and 6.3%, respectively. rGSH levels were assessed using a standard curve method, with absorbance measured at

405nm; the detection range was 2-100 μ M/mL and CVs were 1.9% (intra) and 3.2% (inter). Lipid peroxidation levels were determined by quantifying MDA using the thiobarbituric acid reactive substances (TBARS) assay, with absorbance measured at 532nm; the detection range was 0.38-133.33nM/mL and intra- and inter-assay CVs were 4.9 and 8%, respectively. All absorbance readings were obtained using a spectrophotometer with a 1 cm optical path quartz cuvette (CE1010, Cecil Instruments Limited, Cambridge, UK).

Inflammatory cytokines: The systemic inflammatory profile was characterized by quantifying plasma cytokine concentrations of interleukins (IL-1 β , IL-6 and IL-10) and transforming growth factor beta (TGF- β). The manufacturers' protocols of chicken-specific ELISA kits (CUSABIO, USA) were followed for each assay. Briefly, plasma samples and standards were incubated in antibody-coated microplates with the appropriate HRP-conjugates or biotin-antibodies, followed by sequential incubation with HRP-avidin and TMB substrate. A microplate reader was used the OD value at 450nm. The detection ranges were 0.27-200, 15.6-1000, 1-200 and 0.312-20ng/mL for IL-1 β , IL-6, IL-10 and TGF- β , respectively. Intra- and inter-assay coefficients of variation were below 15% for IL-10 and below 8 and 10% for IL-1 β , IL-6 and TGF- β assays, respectively.

Immunological response: Heparinized blood samples were used to determine total leukocyte count (TLC) and heterophil-to-lymphocyte (H/L) ratio using a hemocytometer and HEMA-3 staining, respectively. Leukocyte cell viability (LCV%) was assessed from peripheral blood mononuclear cells (PBMCs) using a colorimetric assay, with absorbance measured at 570nm and results expressed as a percentage of the control. Humoral immunity was evaluated by intravenously injecting birds with SRBC at 28 days of age, followed by serum collection at day 35. Antibody titers were determined using a hemagglutination assay and expressed as log₂ of the highest dilution showing visible agglutination. Cellular immunity was assessed using the phytohemagglutinin (PHA) wattle swelling test, with responses measured 24h post-injection. Additionally, lymphocyte proliferation was evaluated at the end of the trial using PBMCs stimulated with lipopolysaccharide (B cells) or concanavalin A (T cells). Cell proliferation was assessed using an MTT assay and stimulation indices were calculated as the ratio of absorbance in stimulated cells to that in unstimulated cells.

Intestinal histomorphology and microbial analysis: Jejunum samples were collected from the pre-Meckel's diverticulum area for histomorphological evaluation, including villus height (VH), crypt depth (CD) and the VH/CD ratio. The fixation of paraffin embedded sectioned tissues (\approx 5 μ m) was carried out in 10% formalin followed by staining with eosin and hematoxylin. Morphometric measurements were obtained from multiple sections using light microscopy with digital image analysis. Cecal samples were collected for microbiological analysis. After weighing, cecal contents were homogenized in sterile saline, serially diluted and plated on de Man, Rogosa and

Sharpe (MRS), Xylose-Lysine-Deoxycholate (XLD), or Eosin-Methylene-Blue (EMB) agar (Oxoid, Thermo Fisher Scientific Inc., Hampshire, UK) to enumerate Lactobacillus (LCB), Salmonella (SLM), or Escherichia coli (EC), respectively. Plates for SLM and EC were incubated aerobically at 37°C for 24h, whereas LCB plates were incubated anaerobically at 30°C for 48h. Bacterial counts were expressed as log₁₀ (cfu/g).

Statistical analysis: The pen average served as the experimental unit for productive performance traits, while the individual birds served as the experimental unit for blood, gut and microbial traits. Data were analyzed using SPSS software (version 22.0, IBM Corp., NY, USA, 2013). Normality was verified using the Shapiro-Wilk test prior to analysis. Treatment effects were evaluated by one-way ANOVA and Tukey's post hoc test. Linear and quadratic responses to increasing SBM replacement with BSFLM were assessed using polynomial contrasts and quadratic regression was applied when appropriate to estimate optimal replacement levels. Optimal replacement levels were calculated as $-b/(2c)$ from the fitted quadratic equation ($Y=a+bX+cX^2$). Statistical significance was set at $P<0.05$.

RESULTS

Growth performance: The effect of replacing SBM with BSFLM at increasing rates on the productive performance of broilers is given in Table 4. Replacing SBM with BSFLM significantly improved FBW, BWG and EBI, reaching maximum values at 30% BSFLM inclusion, then declined with higher BSFLM inclusion rates (40-50%), compared to the control. BSFLM inclusion did not affect TFI ($P>0.05$); however, the FCR was better ($P<0.05$) in the 20-30% BSFLM groups than in the 40-50% BSFLM groups. The quadratic regression showed a significant improvement in growth performance with an optimal replacement at approximately 18.9% BSFLM ($FBW=2608.9+9.9X-0.3X^2$, $R^2=0.724$; $BWG=1421.5+9.9X-0.3X^2$, $R^2=0.729$; and $EBI=387.1+5.0X-0.1X^2$, $R^2=0.699$).

Table 4: Effect of soybean meal (SBM) replacement with black soldier fly larvae meal (BSFLM) at incremented rates in the broiler diets on their productive performance traits

Replacement level	IBW (g)	FBW (g)	BWG (g)	TFI (g)	FCR	EBI
0%	1187	2613 ^{bc}	1426 ^{bc}	2446	1.72 ^{bc}	390 ^{bc}
10%	1189	2668 ^{ab}	1480 ^{ab}	2464	1.67 ^{bc}	416 ^{ab}
20%	1188	2701 ^a	1514 ^a	2457	1.62 ^c	436 ^{ab}
30%	1188	2711 ^a	1523 ^a	2466	1.62 ^c	438 ^a
40%	1188	2540 ^{cd}	1352 ^{cd}	2441	1.81 ^{ab}	348 ^{cd}
50%	1187	2464 ^d	1277 ^d	2459	1.94 ^a	312 ^d
SEM	1.9	28.8	28.1	22.1	0.048	15.9
Polynomial contrasts (P-value)						
Linear term	0.782	<0.001	<0.001	0.946	<0.001	<0.001
Quadratic term	0.507	<0.001	<0.001	0.623	<0.001	<0.001
Combined term	0.930	<0.001	<0.001	0.849	<0.001	<0.001
Regression analysis						
R ²	-	0.724	0.729	-	0.654	0.699
P-value	-	<0.001	<0.001	-	<0.001	<0.001

Data represent the means of 6 replicate yards per treatment group and the standard error of means (SEM). Means with different superscript letters within the same column differ significantly ($P<0.05$). Parameters observed are initial body weight (IBW) at 22d of age; final body weight (FBW) at 35d of age; body weight gain (BWG) during 22-35d of age; total feed intake (TFI) during 22-35d of age; feed conversion ratio (FCR) based on TFI/BWG during 22-35d of age and European broiler index (EBI)

Biochemical assay: As shown in Table 5, SBM replacement with BSFLM significantly influenced plasma metabolites. Results demonstrated a significant ($P<0.05$) increase in ft_3 concentration at 10-40% BSFLM replacement rates compared to the control, followed by a decline at 50% replacement. The quadratic regression showed an optimal replacement rate at 29% BSFLM ($ft_3=1.801+0.058X-0.001X^2$, $R^2=0.608$, $P<0.001$). In contrast, plasma AST, ALT, CR and UA levels exhibited a significant ($P<0.05$) quadratic pattern, with optimal reductions at BSFLM rates of 25.4, 26.9, 26.3 and 17.0%, respectively, but with weak coefficient correlation ($AST=50.428-0.407X+0.008X^2$, $R^2=0.217$; $ALT=8.574-0.269X+0.005X^2$, $R^2=0.385$; $CR=0.688-0.003X+0.000X^2$, $R^2=0.267$ and $UA=6.271-0.034X+0.001X^2$, $R^2=0.283$).

Table 5: Effect of soybean meal (SBM) replacement with black soldier fly larvae meal (BSFLM) at incremented rates in the broiler diets on the plasma free iodothyronine and liver and kidney metabolites

Replacement level	ft_3 (ng/mL)	AST (U/mL)	ALT (U/mL)	CR (mg/dL)	UA (mg/dL)
0%	1.72 ^b	48.57 ^{ab}	8.19 ^a	0.68 ^{ab}	6.18 ^a
10%	2.43 ^a	50.78 ^a	7.03 ^{ab}	0.67 ^{ab}	6.10 ^a
20%	2.43 ^a	44.92 ^{ab}	5.87 ^{ab}	0.67 ^{ab}	6.02 ^a
30%	2.37 ^a	42.66 ^b	4.12 ^b	0.64 ^b	5.50 ^b
40%	2.16 ^a	46.59 ^{ab}	7.04 ^{ab}	0.65 ^{ab}	6.00 ^a
50%	1.68 ^b	49.41 ^a	8.69 ^a	0.69 ^a	6.17 ^a
SEM	0.146	1.987	0.996	0.014	0.130
Polynomial contrasts (p-value)					
Linear term	0.241	0.371	0.894	0.821	0.260
Quadratic term	<0.001	0.003	<0.001	0.001	<0.001
Combined term	<0.001	0.003	0.001	0.007	<0.001
Regression analysis					
R^2	0.608	0.217	0.385	0.267	0.283
p-value	<0.001	0.018	<0.001	0.006	0.004

Data represent the means of 6 replicate birds per treatment group and the standard error of means (SEM). Means with different superscript letters within the same column differ significantly ($P<0.05$). Parameters observed are free triiodothyronine (ft_3); aspartate aminotransferase (AST); alanine aminotransferase (ALT), uric acid (UA) and creatinine (CR).

Redox biomarkers: Incremental replacement of SBM with BSFLM significantly influenced plasma redox biomarkers in broilers (Table 6). Compared with the control, TAOC, TSOD and rGSH levels increased progressively with increasing BSFLM inclusion, reaching physiological peaks at 30% replacement, followed by a decline at higher inclusion levels. The regression analysis confirmed significant quadratic responses ($P<0.05$) for the antioxidant activity ($TAOC=4.978+0.476X-0.008X^2$, $R^2=0.732$; $TSOD=6.218+0.183X-0.003X^2$, $R^2=0.578$; $rGSH=25.035+0.578X-0.010X^2$, $R^2=0.684$), with estimated optimal BSFLM replacement rates of 29.8, 30.5 and 28.9%, respectively. In contrast, MDA concentrations decreased as BSFLM inclusion increased, with the lowest values observed at 40% replacement, compared to the control. The polynomial and quadratic regression analyses showed a linear decrease ($p<0.001$) in the MDA with incremental BSFLM replacement rates ($MDA=2.786-0.048X+0.000X^2$, $R^2=0.731$).

Inflammatory cytokines: Incremental replacement of soybean meal with BSFLM significantly modulated plasma inflammatory cytokines in broilers (Table 7). Compared with the control, significant decreases in plasma IL-1 β and significant increases in plasma IL-10 were observed at 20-30% BSFLM. The regression analysis showed a significant quadratic pattern for IL-1 β

and IL-10, with optimal replacement rates of 27.3 and 19.7% BSFLM, respectively ($IL-1\beta=15.267-0.164X+0.003X^2$, $R^2=0.631$; $IL-10=21.472+0.118X-0.003X^2$, $R^2=0.480$; $P<0.001$). Plasma TGF- β was significantly higher at a 30% BSFLM replacement rate compared with the control, whereas IL-6 did not differ among groups. Plasma IL-6 and TGF- β ; however, showed a quadratic regression pattern with weak correlation coefficients ($R^2=0.190$ and 0.291 , respectively).

Table 6: Effect of soybean meal (SBM) replacement with black soldier fly larvae meal (BSFLM) at incremented rates in the broiler diets on the plasma redox biomarkers

Replacement level	TAOC (U/mL)	TSOD (U/mL)	rGSH (μ M/mL)	MDA (nM/mL)
0%	6.05 ^d	6.56 ^c	26.25 ^b	2.67 ^a
10%	6.98 ^{cd}	7.24 ^{bc}	27.47 ^b	2.53 ^a
20%	11.29 ^b	8.02 ^b	32.79 ^a	2.03 ^b
30%	13.47 ^a	9.69 ^a	34.84 ^a	1.77 ^{bc}
40%	11.50 ^b	8.03 ^b	32.12 ^a	1.33 ^c
50%	8.63 ^c	6.96 ^c	28.60 ^b	1.64 ^{bc}
SEM	0.626	0.342	0.931	0.149
Polynomial contrasts (p-value)				
Linear term	<0.001	0.006	<0.001	<0.001
Quadratic term	<0.001	<0.001	<0.001	0.014
Combined term	<0.001	<0.001	<0.001	<0.001
Regression analysis				
R^2	0.732	0.578	0.684	0.731
p-value	<0.001	<0.001	<0.001	<0.001

Data represent the means of 6 replicate birds per treatment group and the standard error of means (SEM). Means with different superscript letters within the same column differ significantly ($P<0.05$). Parameters observed are total antioxidant capacity (TAOC); total superoxide dismutase (TSOD); reduced glutathione (rGSH) and malondialdehyde (MDA).

Table 7: Effect of soybean meal (SBM) replacement with black soldier fly larvae meal (BSFLM) at incremented rates in the broiler diets on the plasma cytokine indices

Replacement level	IL-1 β (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)	TGF- β (pg/mL)
0%	15.08 ^{ab}	153.38	21.46 ^b	218.16 ^b
10%	14.42 ^b	152.07	22.35 ^{ab}	227.61 ^{ab}
20%	13.34 ^c	150.08	22.89 ^a	237.16 ^{ab}
30%	12.62 ^c	150.95	22.93 ^a	264.71 ^a
40%	14.99 ^{ab}	152.83	21.74 ^{ab}	226.39 ^{ab}
50%	15.41 ^a	153.54	21.15 ^b	210.15 ^b
SEM	0.263	1.473	0.417	12.625
Polynomial contrasts (p-value)				
Linear term	0.101	0.65	0.184	0.83
Quadratic term	<0.001	0.012	<0.001	<0.001
Combined term	<0.001	0.148	<0.001	0.003
Regression analysis				
R^2	0.631	0.190	0.480	0.291
p-value	<0.001	0.031	<0.001	0.003

Data represent the means of 6 replicate birds per treatment group and the standard error of means (SEM). Means with different superscript letters within the same column differ significantly ($P<0.05$). Observed parameters includes interleukin-1 β , 6, 10 and transforming growth factor-beta (TGF- β).

Immunological parameters: As shown in Table 8, replacing SBM with BSFLM demonstrated significant immunostimulatory potential. TLC counts were higher in the 30% BSFLM rate than in the control; however, the quadratic pattern was very low ($R^2=0.260$). The H/L ratio decreased as the BSFLM rate increased in broiler diets, showing a quadratic regression with a moderate correlation; the lowest values occurred at a 30% replacement rate ($H/L\ ratio=0.486-0.006X+0.000X^2$, $R^2=0.535$, $P<0.05$). In contrast, LCV, anti-SRBC titers, PHA responses and B- and T-cell stimulation indices were

improved with a markedly quadratic pattern, showing peaks at optimal replacement rates between 21.6-29.8% ($LCV=100.053+1.597X-0.037X^2$, $R^2=0.633$; $anti-SRBC-titers=6.410+0.110X-0.002X^2$, $R^2=0.703$; $PHA-response=0.520+0.011X+0.000X^2$, $R^2=0.608$; $BSI=3.052+0.100X-0.002X^2$, $R^2=0.608$; and $TSI=4.78+0.119X-0.002X^2$, $R^2=0.609$).

Table 8: Effect of soybean meal (SBM) replacement with black soldier fly larvae meal (BSFLM) at incremented rates in the broiler diets on some immunological parameters

Replacement level	TLC ($10^3/mL$)	H/L ratio	LCV (%)	Ab titer (log_2)	PHA test (mm)	B-cells (SI)	T-cells (SI)
0%	55.08 ^b	0.48 ^{ab}	100.00 ^c	6.68 ^d	0.52 ^e	3.05 ^c	4.90 ^b
10%	57.41 ^{ab}	0.45 ^{bc}	109.47 ^b	6.85 ^{cd}	0.60 ^c	3.73 ^b	5.38 ^b
20%	55.80 ^b	0.44 ^{bc}	121.09 ^a	7.68 ^b	0.65 ^b	4.42 ^a	6.37 ^a
30%	60.80 ^a	0.40 ^c	121.43 ^a	8.53 ^a	0.67 ^a	4.43 ^a	6.55 ^a
40%	53.82 ^b	0.48 ^{ab}	92.71 ^c	7.56 ^b	0.56 ^d	3.30 ^{bc}	5.25 ^b
50%	52.99 ^b	0.53 ^a	93.37 ^c	7.01 ^c	0.51 ^f	3.14 ^{bc}	4.88 ^b
SEM	1.601	0.021	2.590	0.063	0.004	0.209	0.239
Polynomial contrasts (p-value)							
Linear term	0.097	0.011	<0.01	<0.001	<0.001	0.513	0.833
Quadratic term	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Combined term	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Regression analysis							
R ²	0.260	0.535	0.633	0.703	0.608	0.608	0.609
p-value	0.007	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Data represent the means of 6 replicate birds per treatment group and the standard error of means (SEM). Means with different superscript letters within the same column differ significantly ($P<0.05$). Parameters observed are total leukocyte cells (TLC); heterophil to lymphocyte ratio (H/L ratio); leukocyte cell viability (LCV, % of control); antibody titer (Ab titer) against sheep red blood cells; phytohemagglutinin reaction (PHA) test and stimulation index (SI).

Intestinal histomorphology and microbial analysis: Microscopic examination of jejunal sections revealed a classical histological architecture in all BSFLM groups (Fig. 1). However, the 50% BSFLM group exhibited mild villous shortening and limited epithelial desquamation, while the jejunal architecture remained largely preserved. Moreover, increasing replacement levels of SBM with BSFLM significantly affected jejunal histomorphology and cecal microbiota (Table 9). The morphometric results showed a significant quadratic trend with peaks in VH and VH/CD ratio at 30% replacement rates and a decline in CD at optimal replacement of 20.8% ($VH=1.908+0.015X+0.000X^2$, $R^2=0.360$; $VH/CD\ ratio=4.705+0.083X-0.002X^2$, $R^2=0.421$; and $CD=0.406-0.004X+0.000X^2$, $R^2=0.611$). The cecal microbiota analysis showed a quadratic increase in LCB counts at optimal replacement rate of 20.5%, while SLM and EC showed a quadratic decrease at optimal replacement rates of approximately 30% ($LCB=8.680+0.041X-0.001X^2$, $R^2=0.379$; $SLM=1.500-0.060X+0.001X^2$, $R^2=0.396$; and $EC=7.317-0.181X+0.003X^2$, $R^2=0.651$).

Table 9: Effect of soybean meal (SBM) replacement with black soldier fly larvae meal (BSFLM) at incremented rates in the broiler diets on jejunal histomorphology and cecal microbiota

Replacement level	VH (mm)	CD (mm)	VH/CD ratio	LCB ($log_{10}\ cfu$)	SLM ($log_{10}\ cfu$)	EC ($log_{10}\ cfu$)
0%	1.98 ^b	0.38 ^c	5.17 ^b	8.77 ^{bc}	1.32 ^{ab}	6.93 ^a
10%	1.90 ^{bc}	0.42 ^{bc}	4.57 ^{bc}	8.88 ^{bc}	1.29 ^b	6.38 ^a
20%	2.00 ^b	0.40 ^c	5.03 ^b	8.97 ^b	1.04 ^c	5.30 ^{bc}
30%	2.26 ^a	0.35 ^d	6.59 ^a	9.48 ^a	0.06 ^d	3.98 ^d
40%	1.81 ^c	0.44 ^b	4.10 ^{cd}	8.75 ^{bc}	1.32 ^{ab}	4.92 ^{cd}
50%	1.75 ^c	0.50 ^a	3.47 ^d	8.55 ^c	1.39 ^a	6.22 ^{ab}
SEM	0.050	0.011	0.218	0.133	0.030	0.310
Polynomial contrasts (p-value)						
Linear term	<0.001	<0.001	<0.001	0.229	0.003	<0.001
Quadratic term	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Combined term	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Regression analysis						
R ²	0.360	0.611	0.421	0.379	0.396	0.651
p-value	0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Data represent the means of 6 replicate samples per treatment group and the standard error of means (SEM). Means with different superscript letters within the same column differ significantly ($P<0.05$). Parameters observed are villus height (VH); Crypt depth (CD); *Lactobacillus* (LCB); *Salmonella* (SLM); *Escherichia coli* (EC).

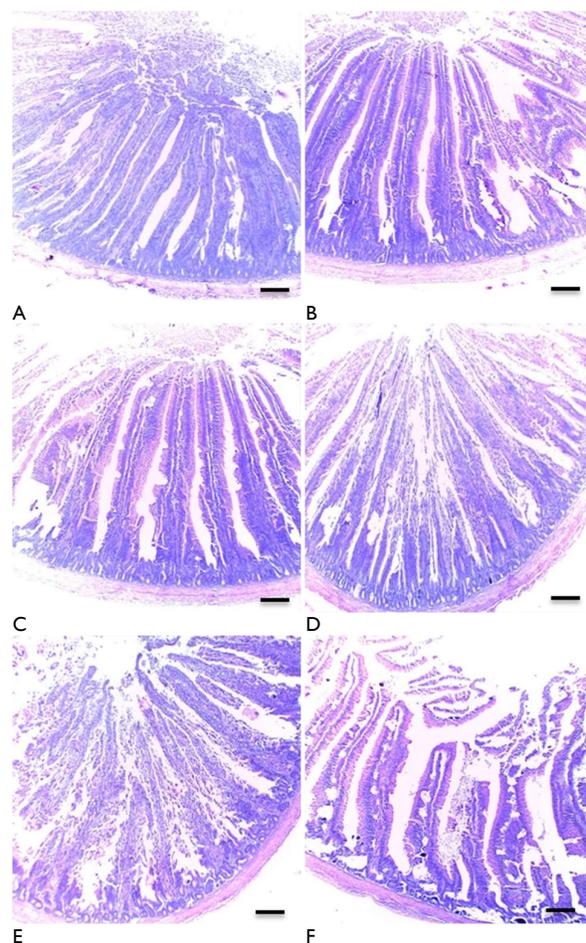


Fig. 1: The microscopic examination of jejunal histology sections obtained from broiler chickens fed diets replacing soybean meal (SBM) with black soldier fly larvae meal (BSFLM) at incremental rates of 0 (panel A), 10 (panel B), 20 (panel C), 30 (panel D), 40 (panel E) and 50% (panel F), respectively (Hematoxylin & eosin, Scale bars = 50 μ m). Jejunal sections showed a classical histological architecture in all BSFLM groups; however, the 50% BSFLM group exhibited mild villous shortening and limited epithelial desquamation, without severe structural damage.

DISCUSSION

The results indicate that replacement levels up to 30% did not impair performance under the present experimental conditions; however, regression analysis suggests that the optimal biological inclusion level is closer to 20%. The improvement of performance traits at moderate inclusion of BSFLM in broiler diets is likely due to its digestible protein, methionine and lysine contents (Baderuddin *et al.*, 2024) and bioactive lipids such as lauric acid (Raju *et al.*, 2024). While dietary ME, calcium and phosphorus concentrations increased slightly with increasing BSFLM inclusion, the magnitude of these differences was modest and unlikely to fully account for the observed quadratic performance pattern. It is worth noting that the improvement in weight gain from BSFLM inclusion was not accompanied by increased feed intake in this study but could be attributed to increased nutrient density and better energy utilization, as reported by several studies (Martínez Marín *et al.*, 2023; Baderuddin *et al.*, 2024). In contrast, higher inclusion levels (40-50%) reduced growth, possibly from amino acid imbalance, increased neutral decongestant fiber (NDF), elevated dietary chitin, or reduced palatability (Seyedalmoosavi *et al.*, 2022; Facey *et al.*, 2023; Tsementzis *et al.*, 2025).

The high levels of plasma fT_3 with BSFLM included at moderate levels (20-30%) in broiler diets may be attributed to the elevated tyrosine concentration in BSFLM relative to SBM (Khaliq *et al.*, 2015) and may enhance metabolic activity and energy utilization, reflecting the improvement of growth efficiency (Huang *et al.*, 2024). Liver and kidney metabolites (ALT, AST, CR, UA) were also optimized at moderate BSFLM inclusion levels compared to the control and high-inclusion groups, supporting hepatic integrity and superior nitrogen utilization efficiency (Chhetri *et al.*, 2025). The higher inclusion levels often worsen renal/hepatic burden indirectly via poor digestibility and metabolic stress induced by chitin loads (Facey *et al.*, 2023; Adam *et al.*, 2024).

Furthermore, the significant increase in the antioxidant status of birds fed 30% BSFLM indicates its consistent effectiveness in increasing antioxidant enzyme activity, lowering lipid peroxidation and providing maximum protection of tissues from oxidative stress. These findings support previous evidence that insect-derived bioactive compounds, including antimicrobial peptides (AMPs), lauric acid and chitin derivatives, exert antioxidant properties. A meta-analysis of AMP trials in broilers reported consistent improvements in antioxidant indicators, including glutathione peroxidase, superoxide dismutase and total antioxidant activity (Sholikin *et al.*, 2021). It was also reported that AMPs indirectly improve redox status via reducing oxidative stress load and improving gut immunomodulatory response (Nazeer *et al.*, 2021). In addition, the BSFLM contents of lauric acid and chitin derivatives contribute to antioxidant capacity by lowering inflammatory and microbial sources of oxidative stress (Osho and Adeola, 2020; Wu *et al.*, 2021). However, the adverse effects of high replacement levels with BSFLM (40-50%) on the antioxidant capacity may be associated with metabolic stress induced by excessive dietary chitin (Adam *et al.*, 2024) or with limitations in sulfur-containing

amino acids required for glutathione (GSH) synthesis (Heuel *et al.*, 2021; Bonomini *et al.*, 2024).

Inflammation in chickens is regulated by pro- and anti-inflammatory cytokines (Muzamil *et al.*, 2021). In this study, moderate BSFLM inclusion (20-30%) promoted an anti-inflammatory immune profile, characterized by increased IL-10 and reduced IL-1 β . The low R^2 values for IL-6 and TGF- β indicate that expression of these cytokines is influenced by multiple non-nutritional factors, such as infection, microbiota, immune stress and environmental factors (Liu *et al.*, 2024; Maghsoudi *et al.*, 2026). Similarly, the TLC results indicate that only about 26% of the variance in TLC was attributable to the BSFLM treatment in the present study, suggesting that BSFLM's effect on TLC may be mainly influenced by other environmental factors. In contrast, a significant correlation (54-70%) of moderate BSFLM treatment was observed with the improvement of other immune indices evaluated in the current study, indicating strengthened immunomodulatory properties of BSFLM (Maghsoudi *et al.*, 2026). Although lowering H/L ratios in the 30% BSFLM group indicates a reduced experience of physiological stress in birds, other research studies have evidenced BSFLM-induced mitigation of other stress indicators, such as corticosterone, rather than H/L ratio (Mazlan *et al.*, 2024). The enhancement of immune parameters, particularly T-cell proliferation, can be mechanistically attributed to bioactive components of BSFLM, such as lauric acid and chitin (de Souza Vilela *et al.*, 2021). Lauric acid promotes NF- κ B signaling pathway and cytokine secretion to stimulate Th1/Th17 differentiation, thereby enhancing humoral and cellular immune responses (Mellouk *et al.*, 2024). Meanwhile, chitin and its oligomers activate innate immune receptors, support dendritic cell maturation and strengthen CD8⁺ T-cell responses (Maia *et al.*, 2023). On the other hand, our findings and those of other poultry studies suggest that higher replacement rates with BSFLM can impair intestinal morphology, induce pronounced inflammation and downregulate immune responses, particularly under pathogenic or physiological stress challenges (Dalmoro *et al.*, 2025).

Gut health is essential for efficient nutrient absorption, immune function and pathogen control in broilers (Cundra *et al.*, 2024). It is well known that full-fat BSFLM delivers more medium-chain fatty acids, including lauric acid, which altered crop and gizzard pH, modify intestinal transit time and accelerate antimicrobial activity (Aslam *et al.*, 2025). The intestinal response to BSFLM showed a curvilinear pattern, with optimal jejunal morphology and cecal microbiota observed at 20-30% SBM replacement. At these levels, increased villus height and reduced crypt depth indicated enhanced absorptive capacity and improved gut development (Dabbou *et al.*, 2018), while favorable shifts in cecal microbiota-particularly an increase in lactobacillus bacteria-suggested a prebiotic effect of BSFLM, likely mediated by lauric acid and antimicrobial peptides (de Souza Vilela *et al.*, 2023). These changes are associated with improved performance and production efficiency (Zaghari *et al.*, 2020). Furthermore, it is documented that increased villus length is typically accompanied by reductions in inflammatory signaling through initiation or normalization of cytokine expression,

including IL-1 β , IL-6, TGF- β and other pro-inflammatory markers (Xu *et al.*, 2021; Comi *et al.*, 2025). In contrast, higher BSFLM inclusion levels disrupted intestinal structure and microbial balance, likely due to excessive chitin, which impaired protein digestibility (Schiavone *et al.*, 2017) and promoted harmful microbial fermentation, thereby impairing growth at 40-50% replacement (Grond *et al.*, 2018).

Overall, the present findings demonstrate that the biological response to dietary BSFLM substitution follows a clear dose-dependent, curvilinear pattern. Moderate inclusion levels (20–30%) consistently supported growth efficiency, endocrine balance, antioxidant defense, immune competence and intestinal structural–microbial integrity, whereas excessive replacement ($\geq 40\%$) compromised several physiological and productive parameters. This indicates that the benefits of BSFLM are not merely nutritional but also mechanistically linked to coordinated modulation of metabolic, redox, immune and gut health pathways. Thus, under the present experimental conditions, BSFLM can be strategically incorporated at moderate levels as a functional and sustainable alternative to soybean meal, with approximately 20-30% substitution representing the optimal biological and productive threshold in broiler finisher diets.

Conclusions: This study concluded that partially defatted BSFLM can effectively replace SBM in broiler finisher diets at inclusion levels up to 30%, yielding consistent improvements in growth performance, feed efficiency, antioxidant capacity, immune response, inflammatory modulation, intestinal morphology and cecal microbial stability. These results highlight BSFLM as a bioactive, sustainable protein alternative with clear practical relevance for commercial poultry production. However, the decline in performance and physiological responses observed at higher inclusion levels (40-50%) indicates a threshold beyond which nutritional constraints may occur, such as an imbalance in the arginine-to-lysine ratio, excessive dietary chitin accumulation and reduced nutrient digestibility. From a sustainability perspective, the successful partial substitution of SBM with BSFLM supports the principles of the circular bioeconomy, in which insect larvae bio convert organic waste streams into high-value protein ingredients, thereby reducing environmental burdens and dependence on conventional feed resources. Future research should investigate multi-phase feeding strategies, strain variability, standardized processing methods, digestibility dynamics and cost-benefit performance, alongside deeper mechanistic exploration of endocrine, immune and microbiome-mediated pathways to validate BSFLM as a scalable solution for sustainable poultry production.

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Authors contribution: AOA, AAA, AA, GFG, NNK, TAE, HMS and AAMA conceived and designed the study and executed the experiment. AOA, AAA, GFG, NNK, TAE, HMS and AAMA validated and analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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