Italian Journal of Animal Science

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/tjas20

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To cite this article: Nur Ain Farhanim Mazlan, Nur Alia Miswan, Sharmila Ahmad, Hasliza Abu Hassim, Eddy Shahriza Jamien, Hew Wei Ee, Suriya Kumari Ramiah & Zulkifli Idrus (2024) Black soldier fly (*Hermetia illucens*) larvae meal for heat-stressed broiler chicken: its effects on gut health, stress biomarkers, and growth performance, Italian Journal of Animal Science, 23:1, 1391-1402, DOI: 10.1080/1828051X.2024.2401444

To link to this article: https://doi.org/10.1080/1828051X.2024.2401444

Published online: 14 Sep 2024.

ITALIAN JOURNAL OF ANIMAL SCIENCE
2024, VOL. 23, NO. 1, 1391–1402
https://doi.org/10.1080/1828051X.2024.2401444

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PRODUCTION PHYSIOLOGY AND BIOLOGY

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Black soldier fly (*Hermetia illucens*) larvae meal for heat-stressed broiler chicken: its effects on gut health, stress biomarkers, and growth performance

Nur Ain Farhanim Mazlan[a], Nur Alia Miswan[a], Sharmila Ahmad[a], Hasliza Abu Hassim[b,c], Eddy Shahriza Jamien[b], Hew Wei Ee[b], Suriya Kumari Ramiah[b] and Zulkifli Idrus[a,b]

[a]Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor, Malaysia; [b]Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, Serdang, Selangor, Malaysia; [c]Department of Veterinary Preclinical Science, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

ABSTRACT

The study determined the effects of feeding black soldier fly larvae meal (BSFLM) on serum corticosterone (CORT), heat shock protein (HSP) 70, d-lactic acid (DLA), diamine oxidase (DAO) levels, gut health and performance in heat-stressed broiler chickens. Three hundred sixty-day-old male broiler chicks were assigned to BSFLM inclusion at 0% (D0) (12 replicate cages with 10 birds/cage), 5% (D5) (12 replicate cages with 10 birds/cage) or 10% (D10) (12 replicate cages with 10 birds/cage). From days 22 to 35, birds from each dietary group were exposed to either heat stress (heated) at $32 \pm 1\,^{\circ}C$ for 6 h (1100 – 1700 h) or $24 \pm 1\,^{\circ}C$ throughout. There were six replicate cages with 10 birds/cage per diet-temperature subgroup. Diet had no significant effect ($p > 0.05$) on growth performance or mortality rate. The CORT of the heated DO chickens were elevated ($p < 0.05$) but not their D5 and D10 counterparts. Diet did not influence HSP70, but the heat challenge increased the expression of the proteins. The unheated D0 birds showed higher ($p < 0.05$) DLA than the other groups. Irrespective of diet, heat exposure elevated DAO. The heat-stressed D5 and D10 birds had lower ($p < 0.05$) caecal *E.coli* and *Clostridium* spp. than the D0 group. The D10 chickens showed increased caecal *Lactobacillus* spp. counts compared to the other groups. Heat exposure but not diet was detrimental to intestinal morphology. In conclusion, BSFLM as a feed ingredient may support optimum growth performance and enhance gut health and heat tolerance in broilers.

HIGHLIGHTS

- Feeding broilers with black soldier fly larvae meal (BSFLM) of up to 10% reduced heat stress and pathogenic intestinal bacteria count.
- Replacing soybean meal with 10% BSFLM had no adverse effect on the growth performance of broilers.

ARTICLE HISTORY
Received 12 June 2024
Revised 1 September 2024
Accepted 2 September 2024

KEYWORDS
Black soldier fly larvae meal; heat stress; gut health; stress biomarkers; broiler chickens

Introduction

Insect-based diets, compared to human-edible crops such as soybean, offer sustainable and eco-friendly practices for industrial-scale poultry production (Heines et al. 2022; Raman et al. 2022), as insect farming requires less land, water, and feed inputs (Makkar et al. 2014; Stelios et al. 2024). Black soldier fly (*Hermetia illucens*) larvae can convert organic waste into high-quality protein, aiding the circular economy in the poultry industry (Danieli et al. 2011; Makkar et al. 2014; Barragan-Fonseca et al. 2017). These larvae provide a sustainable supply of protein, fat, and natural antioxidants like vitamins E and C (Zulkifli et al. 2022; Torres-Castillo and Olazarán-Santibáñez 2023). Black soldier fly larvae meal (BSFLM) is a potential protein source for poultry feed due to its high protein (37% to 63% dry matter) and fat content (7-29% dry matter), along with essential amino acids, vitamins and minerals (such as iron, calcium, phosphorous, zinc) (Spranghers et al. 2017; Dabbou et al. 2018; Abd El-Hack et al. 2020).

Insect larvae thrive in manure and organic waste, producing antimicrobial peptides that might benefit poultry feed (Elahi et al. 2022). BSFLM can enhance broiler gut health by improving intestinal integrity and caecal

CONTACT Zulkifli Idrus zulidrus@upm.edu.my

microbiota profiles (Biasato et al. 2020). It also increased *Lactobacillus* spp. and reduced pathogenic bacteria like *E. coli* in the caeca due to its antimicrobial properties (Lieberman et al. 2006; Van Huis 2020; Al-Qazzaz et al. 2021). Chitin in insect exoskeletons can inhibit the growth of *E. coli* and *Salmonella* spp. in broiler chickens (Khempaka et al. 2011; Menconi et al. 2014). Beller et al. (2024) reported that *Lachnoclostridium*, a beneficial microorganism that inhibited pathogens and reduced inflammation, was abundant, while harmful pathogens like *Shuttleworthia* and *Oscillibacter*, associated with intestinal infections and inflammation, were reduced in broilers fed diets with 15% BSFLM.

Although previously insects were mainly appreciated for their protein and energy supply, recent work by Stelios et al. (2024) suggested that diets supplemented with *Tenebrio molitor*, *Hermetia illucens* or *Zophobas Morio* larvae enhanced the health and resilience of broiler chickens exposed to mild heat stress (HS). High ambient temperatures pose significant challenges to the poultry industry, causing economic and productivity losses in broiler chicken production. These stressors activate the hypothalamic-pituitary-adrenal (HPA) axis, leading to the secretion of corticosterone, a biomarker for stress in poultry (Tilbrook and Fisher 2020). Studies show that heat exposure consistently elevates corticosterone levels (Najafi et al. 2015; Quinteiro-Filho et al. 2017; Ramiah et al. 2019), which in turn increases the expression of heat shock protein 70 (Kang and Shim 2021; Khondowe et al. 2021), a critical marker of HS in chickens. Heat stress triggers physiological responses such as inflammation, compromising intestinal integrity and causing leaky gut syndrome (Karl et al. 2018; Elnesr and Abdel-Azim 2023; Stelios et al. 2024). It also impairs intestinal morphology (He et al. 2018; Li et al. 2018) and alters the gut bacteria profile (Wang et al. 2018), increasing pathogenic bacteria and reducing beneficial ones, leading to intestinal disorders (Pourabedin and Zhao 2015). Therefore, this study aimed to investigate the effects of partial substitution of soybean meal (SBM) with BSFLM on serum levels of corticosterone (CORT), heat shock protein (HSP) 70, d-lactic acid (DLA), and diamine oxidase (DAO), duodenal villi length and crypt depth, caecal *E. coli, Clostridium* spp., *Salmonella* spp. and *Lactobacillus* spp. counts, and growth performance in heat-stressed broiler chickens. Increased DLA and DAO are commonly associated with intestinal mucosal integrity injury, which could serve as markers of intestinal mucosal barrier integrity and injury in poultry (Wang et al. 2020; Hosseindoust et al. 2022). We hypothesised that partial replacement of BSFLM in the diet can alleviate the negative effect of HS on broilers' intestinal permeability, intestinal morphology, and caecal microbiota counts.

Materials and methods

All animal care procedures were approved by the Institutional Animal Care and Use Committee of the Universiti Putra Malaysia (UPM/IACUC/AUP-U045/2023).

Birds and housing

Three hundred and sixty-day-old male Cobb 500 chicks were purchased from a local hatchery. The chicks were randomly assigned in groups of ten to 36 battery cages with wire floors. Each cage measured 116 (width) × 90 (length) × 50 (height) cm^3. The cages were in six identical environmentally controlled rooms (6 cages per room). The ambient temperature on day 1 was set at 32 ± 1 °C and gradually decreased until 23 ± 1 °C was reached by day 21. The average relative humidity during the experimental period ranged between 65% and 87%. The lighting durations provided were continuous for the first two days, and from day 3 onwards, lighting was 18 h per day. The birds were vaccinated against Newcastle disease and infectious bronchitis on days 7 and 21.

Experimental diet

Starter (days 1–21) and finisher (days 22–35) diets (mash form) were prepared by including, as a feed basis, three levels of BSFLM (0, 5%, and 10%; D0, D5 and D10, respectively) (Table 1). There were 12 replicate cages for each dietary group. The defatted BSFLM (62.20% crude protein and 6.20% crude fat) was obtained from a local commercial producer (Life Origin, Seri Kembangan, Selangor, Malaysia). The larvae were raised on soybean, coconut, and bakery waste. All diets were isocaloric and isonitrogenous, meeting or exceeding the nutrient requirements of Cobb 500 broilers (Cobb-Vantress Inc., Siloam Springs, AR, USA, 2022). The diets were formulated based on the coefficients for standardised ileal digestible amino acids and the apparent metabolisable energy values obtained from previous studies (De Marco et al. 2015; Schiavone et al. 2017; Mwaniki and Kiarie 2018).

Heat treatment

From days 22 to 35, six cages of chickens from each dietary group were exposed to an ambient

Table 1. Experimental diet composition (g/kg as-fed basis).

	Starter (days 1 to 20)			Finisher (days 21 to 35)		
Diets	D0	D5	D10	D0	D5	D10
Ingredients						
Corn	549	538	526	593	596	594
Wheat Bran	21.7	41.0	42.6	44.9	52.3	51.2
Soybean meal	354	295	257	279	222	179
BSFLM	0.000	50.0	100	0.000	50.0	100
Palm Oil	37.0	37.0	34.5	51.0	46.5	42.0
Limestone	12.0	12.0	12.0	10.5	10.5	10.5
Dicalcium Phosphate	16.0	16.5	17.0	12.5	13.0	13.8
L-Threonine	0.600	0.500	0.500	0.000	0.000	0.000
Common Salt	2.50	2.50	2.50	2.50	2.50	2.50
Vitamin Premix[1]	0.400	0.400	0.400	0.400	0.400	0.400
Mineral Premix[2]	1.00	1.00	1.00	1.00	1.00	1.00
DL-Methionine	1.45	1.45	1.35	1.40	1.40	1.40
L-Lysine HCl	1.55	2.20	2.30	1.20	1.90	2.10
Choline Cl −70%	0.500	0.500	0.500	0.500	0.500	0.500
Antioxidant	0.200	0.200	0.200	0.200	0.200	0.200
Sodium Bicarbonate	2.00	2.00	2.00	1.80	1.80	1.80
Calculated						
Energy (Kcal/kg)	2975	2975	2975	3100	3100	3100
Crude protein (%)	21.0	21.6	22.7	18.4	18.98	19.97
Crude fat (%)	6.14	6.45	6.47	7.71	7.60	7.44
Calcium (%)	0.910	0.910	0.910	0.77	0.77	0.77
Total phosphorus (%)	0.710	0.690	0.680	0.63	0.61	0.60
Available phosphorus (%)	0.460	0.460	0.450	0.38	0.38	0.38
Lysine (%)	1.22	1.22	1.22	1.02	1.02	1.02
Methionine (%)	0.460	0.460	0.460	0.420	0.420	0.420
Tryptophan (%)	0.850	0.890	0.960	0.730	0.770	0.840

Note: BSFLM, black soldier fly larvae; D0, 0% BSFLM; D5, 5% BSFLM; D10, 10% BSFLM.
[1]Vitamin Premix provided the following nutrients per kg diet: vitamin A, 24,000 IU; vitamin D_3. 6,000 IU; vitamin E, 80 mg; vitamin K_3, 4 mg; vitamin B_1, 4 mg; vitamin B_2, 10 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.04 mg; niacin, 80 mg; pantothenic acid, 20 mg; folic acid, 2 mg; biotin, 0.3 mg.
[2]Mineral Premix provided the following nutrients per kg diet: Fe, 176 mg; Cu, 145.2 mg; Zn, 120 mg; Mn, 132 mg; I, 1.98 mg; Co, 0.66 mg; Se, 0.44 mg.

temperature of 32 ± 1 °C for 6 h (1100 – 1700 h). Relative humidity was not controlled but ranged from 70 to 80%. The calculated temperature humidity index (THI) (National Oceanic and Atmospheric Administration, 1976) was 78. The time taken for the ambient temperature to rise from 24 to 32 °C was about 30 min. Feed and water were provided *ad libitum* throughout heat treatment. The remaining birds remained at 23 ± 1 °C throughout.

Growth performance

Body weight and feed intake (cage basis) data were recorded on days 1, 21, and 35. Feed conversion ratios (FCR) (feed/gain) were calculated accordingly after adjustment on mortality. Mortality was recorded upon occurrence daily.

Sampling

On Day 35, two birds per cage (12 birds per diet-temperature subgroup) were randomly selected, weighed, and slaughtered according to the halal method (Farouk et al. 2014). Blood samples were collected and centrifuged at 4000 × *g* at 4 °C for 20 min. The harvested serum samples were stored at −80 °C to determine CORT, HSP70, DAO, and DLA. For measurement of intestinal morphology, approximately 5 cm of the middle portion of the duodenum (apex section) was cut, gently flushed with 0.9% saline solution and placed in 10% neutral-buffered formalin.

Blood parameters

According to manufacturer recommendations, CORT was determined using commercially available high-sensitivity EIA kits (AC-15F1, IDS, Boldon, UK). Cross-reactivity of the CORT antiserum was less than 6.7% and 7.8%, respectively, and the detection limit was 27 ng/mL. The HSP70 determination was performed using a commercial ELISA kit specific to chicken (Cat. No. 201-16-0033, Shanghai Sunred Biological Technology, Shanghai, China) according to manufacturer recommendations. All samples were run in the same assay to prevent inter-assay variability. According to the manufacturer's instructions, the DAO and DLA were measured using an ELISA Assay kit

Table 2. Bacteria, primer sequences, and annealing temperatures[1].

Bacteria	Primer Sequence (5′-3′)	Annealing temperature (°C)
Lactobacillus spp.	F: CAT CCA GTG CAA ACC TAA GAG R: GAT CCG CTT GCC TTC GCA	58
Clostridium spp.	F: GAG TTT GAT CMT GGC TCA G R: CCC TTT ACA CCC AGT AA	60
Escherichia coli	F: GTG TGA TAT CTA CCC GCT TCG C R: AGA ACG CTT TGT GGT TAA TCA GGA	60
Salmonella spp.	F: TCG TCA TTC CAT TAC CTA CC R: AAA CGT TGA AAA ACT GAG GA	50

Primers and annealing temperatures were according to Navidshad et al. (2012) and (Rezaei et al. 2015).

(Sunlong Biotech, China) and the EnzyChrom™ D-Lactate assay kit (EDLC-100, BioAssay Systems, USA).

Gut histomorphometric

The fixed duodenum was washed, dehydrated in an automatic tissue processor (Leica TP 1020, Japan), and embedded in paraffin wax (Leica EG 1159, Japan). Sections 5 μm thick were cut using a microtome Jung Multicut (Leica RM 2045, Japan), placed on a glass slide, heated at 57 °C, dried, and stained with haematoxylin and eosin. The histological parameters analysed were villus height (VH), crypt depth (CD), and VH:CD ratio. The VH and CD of each section on slides were analysed using ImageFocus. The VH:CD ratios were then calculated.

Caecal bacteria quantification

The caecal microbiota was quantified following the method by Navidshad et al. (2012). The QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to extract the total DNA following the manufacturer's protocol. The concentration and integrity of isolated DNA were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The cDNA synthesis was performed by reverse transcription (RT) by applying QuantiNova™ SYBR SYBR Green PCR kit (Qiagen, Hilden, Germany). A total of 20 μL reaction mixture was prepared by adding 8.5 μL of 2X SYBR Green PCR Master Mix, 1 μL of each of forward and reverse primers, 1 μL of DNA samples, and 8.5 μL of RNase-free water. The qPCR was performed using BioRad CFXConnect (Biorad, USA), following the manufacturer's protocols. The PCR conditions used were denaturation (95 °C for 10 s), annealing (25 s), and elongation (72 °C for 10 s), which were repeated 40 times in each qPCR reaction. The targeted bacteria, primer sequences, and annealing temperatures are presented in Table 2.

Statistical analysis

All analyses were carried out using SAS software (SAS, 2002). Pens served as the experimental units for growth performance data, while individual birds served as the experimental units for blood parameters, intestinal morphology, and microbial population. Growth performance parameters from days 1 to 21 were analysed by one-way ANOVA, with diet as the main effect of the analysis. A two-way ANOVA was used to determine the main effects of diet and temperature and their interactions on growth performance (days 22 to 35 and days 1 to 35), blood parameters, bacterial population, and intestinal morphometric indices data. When interactions between main effects were significant, comparisons were made within each experimental variable. Duncan's multiple range test was used to separate the means when significant effects were noted. A chi-square test analysed mortality data. Statistical significance is considered at $p \leq 0.05$.

Results

Growth performance and

Diet had no significant effect on feed intake, weight gain, and FCR during the starter period (days 1–21) (Table 3). There was no mortality from days 1 to 21. There were no significant diet x temperature interactions for growth performance during the finisher (days 22 to 35) and overall (days 1 to 35) periods (Table 4). The heat exposure resulted in poorer weight gain, feed intake, and FCR during days 22 to 35 and 1 to 35 compared to the unheated birds. The growth performance of birds during the finisher and overall periods was not significantly affected by diet. The mortality rates of the D0-unheated (0.00%), D0-heated (3.33%), D5-unheated (1.67%), D5-heated (0.00%), D10-unheated (0.00%), and D10-heated (1.67%) broilers during the heat exposure period (days 22 to 35) did not differ significantly ($p > 0.05$).

Table 3. Effect of diet[1] on the growth performance in broiler chickens[2] from days 1 to 21 (Mean ± SEM).

	Diet			
Parameters	D0	D5	D10	p-value
Feed intake, g/bird	1313 ± 17.2	1352 ± 15.4	1324 ± 16.1	0.250
Weight gain, g/bird	919 ± 12.3	911 ± 7.33	893 ± 20.1	0.410
FCR, feed/gain	1.28 ± 0.015	1.33 ± 0.019	1.33 ± 0.020	0.100

[a,b]Means within a row-subgroup with no common letters differ at ($p \leq 0.05$).
[1]D0, 0% black soldier fly larvae meal (BSFLM); D5, 5% BSFLM; D10, 10% BSFLM.
[2]Growth performance data (cage basis) were measured in 12 cages of chickens per dietary group.
FCR: Feed conversion ratio.

Table 4. Effect of diet[1] and temperature[2] on the growth performance in broiler chickens[3] from days 1 to 35 (Mean ± SEM).

	Diet			Temperature				
Parameters	D0	D5	D10	p-value	**Heated**[2]	Diet	Temperature	Diet x Temperature
Feed intake, g/bird								
22-35	2227 ± 41.5	2223 ± 45.2	2226 ± 50.9	2334 ± 23.1[a]	2108 ± 17.2[b]	0.994	< 0.0001	0.839
1-35	3462 ± 40.6	3494 ± 44.0	3470 ± 63.2	3570 ± 29.2[a]	3377 ± 25.0[b]	0.770	< 0.0001	0.801
Weight gain, g/bird								
22-35	1261 ± 29.2	1308 ± 38.9	1278 ± 58.0	1370 ± 23.1a	1193 ± 19.4[b]	0.400	< 0.0001	0.459
1-35	2180 ± 33.0	2219 ± 38.1	2171 ± 68.8	2290 ± 21.7[a]	2093 ± 22.2[b]	0.398	< 0.0001	0.244
FCR, feed/gain								
22-35	1.77 ± 0.026	1.70 ± 0.022	1.75 ± 0.054	1.71 ± 0.024[b]	1.77 ± 0.023[a]	0.199	0.018	0.143
1-35	1.56 ± 0.012	1.54 ± 0.011	1.57 ± 0.031	1.53 ± 0.010	1.58 ± 0.012	0.486	0.001	0.176

[a,b]Means within a row-subgroup with no common letters differ at ($p \leq 0.05$).
[1]D0, 0% black soldier fly larvae meal (BSFLM); D5, 5% BSFLM; D10, 10% BSFLM.
[2]From days 22 to 35, birds from each dietary group were exposed to either heat stress (heated) at 32 ± 1 °C for 6 h (1100 -1700 h) or 24 ± 1 °C throughout (unheated).
[2]Growth performance data (cage basis) were measured in 6 cages of chickens per diet-temperature subgroup.
FCR: Feed conversion ratio.

Table 5. Effect of diet[1] and temperature[2] on serum corticosterone (CORT), heat shock protein (HSP) 70, D-lactic acid (DLA) and diamine oxidase (DAO) levels in broiler chickens[3] on day 35 (Mean ± SEM).

	Diet			Temperature		p-value		
Parameters	D0	D5	D10	Unheated	**Heated**[2]	Diet	Temperature	Diet × Temperature
CORT (ng/mL)	1.82[a]±0.517	0.886[b] ± 0.094	0.78[b] ± 0.113	0.704[b] ± 0.070	1.583[a] ± 0.340	0.006	0.001	0.002
HSP70 (ng/mL)	232 ± 31.4	251.9 ± 33.0	210 ± 23.3	185[b] ± 11.7	286[a] ± 30.8	0.783	0.008	0.131
DLA (mM)	2.21 ± 0.354	1.69 ± 0.331	2.20 ± 0.388	2.21 ± 0.288	1.87 ± 0.294	0.285	0.173	0.032
DAO (ng/mL)	7.16 ± 0.02	7.15 ± 0.02	7.17 ± 0.01	7.13[b] ± 0.01	7.19[a] ± 0.01	0.563	0.0004	0.312

[a,b]Means within a row-subgroup with no common letters differ at ($p \leq 0.05$).
[1]D0: 0% black soldier fly larvae meal (BSFLM); D5:5% BSFLM; D10: 10% BSFLM.
[2]From days 22 to 35, birds from each dietary group were exposed to either heat stress (heated) at 32 ± 1 °C for 6 h (1100 -1700 h) or 24 ± 1 °C throughout (unheated).
[3]Blood samples were collected from 12 birds per diet-temperature subgroup.

CORT and HSP70

There were significant diet x heat treatment interactions ($p = 0.002$) for CORT (Table 5). Diet had no significant effect on the CORT of unheated birds. The heat challenge elevated the CORT of the D0 birds considerably but had a negligible effect on their D5 and D10 counterparts (Table 6). The heat challenge significantly increased ($p = 0.008$) HSP70, but diet did not significantly ($p = 0.783$) affect the protein level (Table 5).

DLA and DAO

There were significant diet x heat treatment ($p = 0.032$) interactions for DLA (Table 5). Under unheated conditions, DLA was significantly increased in the D0 birds compared with the D5 and D10 groups (Table 5). However, following the heat challenge, diet did not significantly affect DLA. Irrespective of diet, the heated birds had significantly ($p = 0.0004$) higher DAO than those of the unheated group (Table 5). Diet had a negligible ($p = 0.563$) influence on DAO.

Caecal microbiota

The diet x temperature interactions for caecal *E.coli* and *Clostridium* spp. counts were significant ($p = 0.002$) (Table 7). The D5 and D10 birds had significantly lower caecal *E.coli* and *Clostridium* spp. counts than their D0 counterparts under heated conditions

Table 6. Mean (±SEM) serum corticosterone (CORT) and d-lactic acid (DLA) levels where diet[1] x temperature[2] interactions were significant in broiler chickens[3].

	CORT (ng/mL)		DLA (mM)	
Diet	Unheated	Heated	Unheated	Heated
D0	$0.636^{bx} \pm 0.105$	$3.00^{ax} \pm 0.825$	$3.21^{ax} \pm 0.458$	$1.40^{bx} \pm 0.386$
D5	$0.584^{ax} \pm 0.073$	$0.929^{ay} \pm 0.181$	$1.44^{ay} \pm 0.273$	$1.89^{ax} \pm 0.569$
D10	$0.869^{ax} \pm 0.143$	$0.907^{ay} \pm 0.125$	$2.09^{ay} \pm 0.581$	$2.29^{ax} \pm 0.544$

[a,b]Means within a row with no common letters differ at ($p \leq 0.05$).
[x,y]Means within a column with no common letters differ at ($p \leq 0.05$).
[1]D0, 0% black soldier fly larvae meal (BSFLM); D5, 5% BSFLM; D10, 10% BSFLM.
[2]From days 22 to 35, birds from each dietary group were exposed to either heat stress (heated) at 32 ± 1 °C for 6 h (1100–1700 h) or 24 ± 1 °C throughout (unheated).
[3]Blood samples were collected from 12 birds per diet-temperature subgroup.

Table 7. Effect of diet[1] and temperature[2] on the caecal bacteria counts in broiler chickens[3] on day 35.

	Diet			Temperature		p-value		
Bacteria (log10 cells/gr)	D0	D5	D10	Unheated	**Heated**[2]	Diet	Temperature	Diet x Temperature
Escherichia coli	$6.01^{a} \pm 0.174$	$5.72^{ab} \pm 0.107$	$5.42^{b} \pm 0.167$	5.72 ± 0.129	5.71 ± 0.131	0.016	0.961	0.002
Clostridium spp.	7.30 ± 0.093	7.21 ± 0.123	6.99 ± 0.130	7.24 ± 0.091	7.09 ± 0.101	0.130	0.263	0.006
Lactobacillus spp.	$5.54^{b} \pm 0.191$	$5.46^{b} \pm 0.184$	$6.22^{a} \pm 0.193$	5.85 ± 0.145	5.63 ± 0.179	0.012	0.327	0.794
Salmonella spp.	ND	ND	ND	ND	ND	–	–	–

[a,b]Means within a row-subgroup with no common letters differ at ($p \leq 0.05$).
[1]D0, 0% black soldier fly larvae meal (BSFLM); D5, 5% BSFLM; D10, 10% BSFLM.
[2]From days 22 to 35, birds from each dietary group were exposed to either heat stress (heated) at 32 ± 1 °C for 6 h (1100 -1700 h) or 24 ± 1 °C throughout (unheated).
[3]Caecal content was collected from 12 birds per diet-temperature subgroup.
ND, not detected.

Table 8. Mean (±SEM) caecal *E.coli* and *Clostridium* spp. counts where diet[1] x temperature[2] interactions were significant in broiler chickens.[3]

Bacteria count (log10 cells/gr)	*E. coli*		*Clostridium* spp.	
Diet	Unheated	**Heated**[2]	Unheated	**Heated**[2]
D0	$5.59^{bx} \pm 0.231$	$6.44^{ax} \pm 0.201$	$7.08^{bx} \pm 0.147$	$7.52^{ax} \pm 0.074$
D5	$5.88^{ax} \pm 0.163$	$5.56^{ay} \pm 0.130$	$7.44^{ax} \pm 0.044$	$6.97^{ay} \pm 0.228$
D10	$5.70^{ax} \pm 0.273$	$5.15^{ay} \pm 0.168$	$7.19^{ax} \pm 0.221$	$6.79^{ay} \pm 0.118$

[a,b]Means within a row-subgroup with no common letters differ at ($p \leq 0.05$).
[x,y]Means within a column with no common letters differ at ($p \leq 0.05$).
[1]D0, 0% black soldier fly larvae meal (BSFLM); D5, 5% BSFLM; D10, 10% BSFLM.
[2]From days 22 to 35, birds from each dietary group were exposed to either heat stress (heated) at 32 ± 1 °C for 6 h (1100 -1700 h) or 24 ± 1 °C throughout (unheated).
[3]Caecal content was collected from 12 birds per diet-temperature subgroup.

(Table 8). However, the caecal *E.coli* and *Clostridium* spp. counts of the unheated birds were not affected by diet. The D10 chickens had significantly ($p = 0.012$) higher caecal *Lactobacillus* spp. count than the D0 and D5 groups. Temperature had a negligible ($p = 0.263$) effect on caecal *Lactobacillus* spp. count. Irrespective of diet and temperature, *Salmonella* spp. was not detected in the caecal content.

Intestinal morphology

Table 9 shows no significant ($p = 0.672$) diet x temperature interactions for duodenal VH, CD, and VH:CD ratio. However, HS significantly reduced the VH ($p < 0.001$) and VH:CD ratio ($p = 0.003$) of the duodenum but had no significant ($p = 0.095$) effect on CD.

Discussion

Many researchers have reported that heat stress can impair the production performance of broilers by decreasing feed intake, weight gain, and feed efficiency (Al-Batshan and Hussein 1999; Awad et al. 2020; Kang and Shim 2021). Poultry exposed to elevated environmental temperatures showed behavioural, physiological, and immunological reactions that negatively impact their productivity (He et al. 2018; Nawab et al. 2018). The present findings indicated that six hours of exposure to 32 ± 1 °C from days 22 to

Table 9. Effect of diet[1] and temperature[2] on the intestinal histomorphology in broiler chickens[3] on day 35.

	Diet			Temperature		p-value		
Parameters	D0	D5	D10	Unheated	**Heated**[2]	Diet	Temperature	Diet x Temperature
Villi Height (μm)	855 ± 23.8	864 ± 19.5	833 ± 21.3	903[a] ± 15.90	798.84[b] ± 14.53	0.510	<.0001	0.672
Crypt Depth (μm)	73.4 ± 1.29	77.9 ± 1.67	78.1 ± 2.15	74.8 ± 1.53	78.1 ± 1.33	0.095	0.097	0.381
Villi Height: Crypt Depth	11.3 ± 0.391	10.7 ± 0.447	10.9 ± 0.599	11.8[a] ± 0.337	10.2[b] ± 0.204	0.572	0.003	0.741

[a,b]Means within a row-subgroup with no common letters differ at ($p \leq 0.05$).
[1]D0, 0% black soldier fly larvae meal (BSFLM); D5, 5% BSFLM; D10, 10% BSFLM.
[2]From days 22 to 35, birds from each dietary group were exposed to either heat stress (heated) at 32 ± 1 °C for 6 h (1100 -1700 h) or 24 ± 1 °C throughout (unheated).
[3]Duodenal samples were collected from 12 birds per diet-temperature subgroup.

35 was detrimental to broilers' feed intake, weight gain, and FCR. Our results concur with Najafi et al. (2015) that the cyclic HS did not affect the mortality rate of broilers.

Black soldier fly larvae meal represents an alternative protein source and has been utilised in poultry, swine, and aquaculture diets to substitute fish or soybean meal. Recent literature shows that the inclusion of 5 to 20% BSFLM in broiler diet supported optimum feed intake, weight gain, and FCR of broiler chickens (de Souza Vilela et al. 2021; Seyedalmoosavi et al. 2022; Fruci et al. 2023). Under the current experimental conditions, BSFLM could replace soybean meal by up to 10% in the starter and finisher diets without any detrimental effect on the growth performance of unheated and heated broilers. However, (Murawska et al. 2021) reported that replacing soybean meal with 50 to 75% or 100% of BSFLM in the diets of broiler chickens had adverse effects on growth performance, carcase quality, and some meat characteristics. The authors suggested that the high chitin content in the diet, which is not readily digestible by monogastric animals and can negatively affect protein digestibility, may have accounted for the depressed growth performance of the broilers fed higher levels of BSFLM. On the contrary, soybean-based laying hen feeds can be entirely replaced by BSFLM without adversely affecting productivity (Heuel et al. 2021). It was reported that the amino acid digestibility and AME of some feed ingredients in broilers and layers could differ due to age and genotype differences (Huang et al. 2007; Adedokun et al. 2009).

It is well established that heat stress activates the chicken HPA activity, increasing blood corticosterone levels and consequent deterioration in birds' performance and well-being (Quinteiro-Filho et al. 2010). In this study, the D0 birds, as measured by CORT, were more distressed than their D5 and D10 counterparts following heat exposure. It is interesting to note that the CORT of heated and unheated D5 and D10 chickens were similar. Thus, it appears that dietary incorporation of BSFLM at 5% or 10% enhanced the ability of broilers to withstand higher temperatures. The beneficial effects of BSFLM on heat tolerance could be associated with its rich content of various bioactive compounds, including vitamins E and C, carotenoids, and phenolic compounds (Zulkifli et al. 2022) (El-Sayed et al. 2022; Torres-Castillo and Olazarán-Santibáñez 2023; Stelios et al. 2024). Earlier research showed a synergistic impact of dietary vitamins E and C on the antioxidant levels (Jena et al. 2013) and productivity (Ipek and Dikmen 2014) of heat-stressed poultry. Gopi et al. (2022) reported that the antioxidant properties of polyphenols contribute to enhancing body weight gain and meat quality in broiler chickens while also alleviating the detrimental effects of heat stress. This was achieved by diminishing the production of reactive oxygen species, thereby reducing oxidative stress. The superior amino acid profile and digestibility of BSFLM (De Marco et al. 2015; Schiavone et al. 2017) may have also accounted for the improved heat tolerance in the D5 and D10 chickens. The profound impact of protein and amino acid nutrition on heat-stressed chickens has been documented (Gonzalez-Esquerra and Leeson 2005). The enhanced heat tolerance in the D5 and D10 birds could also be associated with the prebiotic effect of chitin (Kipkoech et al. 2023). Chitin, the main fibrous compound in arthropod exoskeletons, is a polysaccharide composed of *N*-acetyl-2-amino-2-deoxyglucose (GlcNAc) units linked by β-(1→4) bonds (Lopez-Santamarina et al. 2020). The benefits of dietary prebiotic supplementation in alleviating heat stress in poultry have been well documented (Awad et al. 2021; Ringseis and Eder 2022).

The data presented here confirm previous findings that heat stress augmented HSP70 expression in chickens (Najafi et al. 2015; Kang and Shim 2021; Khondowe et al. 2021). El-Sayed et al. (2022) exposed Japanese quail to 40 ± 2 °C for 8 h daily for seven days and noted reduced heat shock protein 70 gene transcription when the birds were fed a diet containing 50% and 100% BSFLM as a source of protein. Based on the heat shock protein 70 gene transcription, the

authors concluded that feeding birds BSFLM as the source of protein could alleviate the detrimental effects of heat stress in quail. On the contrary, in the present study, the D5 and D10 diets had a negligible impact on HSP70. The discrepancies in results might be partly due to the lower levels of inclusion of BSFLM and a milder heat challenge in our study, in contrast to those conducted by El-Sayed et al. (2022).

Maintaining normal morphometric characteristics, intestinal barrier function, and intestinal permeability are critical for preventing the translocation of bacteria from the gut, proper digestion and absorption of nutrients, and consequently, optimum growth and general well-being of poultry (Burkholder et al. 2008; Quinteiro-Filho et al. 2010). When the intestinal mucosal barrier is damaged, it will evoke self-protection and repair processes, including releasing some amino acids, short-chain fatty acids, cytokines, and other substances (Huda-Faujan et al. 2010; Rao and Samak 2012). Diamine oxidase is found exclusively in the lumen of the small intestine, whereas DLA is the metabolite of bacteria in the gut (Wang et al. 2020; Hosseindoust et al. 2022). Previous studies showed that heat exposure led to a considerable surge in DLA and DOA in broiler chickens (Lan et al. 2020; Alhotan et al. 2021; Peng et al. 2023). In the present study, heated birds showed elevated DAO irrespective of diet. We noted that the unheated D5 and D10 chickens had lower DLA than their D0 counterparts, suggesting that BSFLM inclusion could help maintain the integrity of the intestinal mucosal barrier. However, following the heat challenge, all birds had similar DLA.

In consonant with many previous studies (Song et al. 2013; Varasteh et al. 2015; Zhang et al. 2017; Awad et al. 2018; Nanto-Hara et al. 2020), we noted that heat exposure reduced intestinal VH and VH:CD ratio in chickens. Heat stress may induce severe intestinal microarchitecture modifications, reducing broilers' villi height (Song et al. 2014; Santos et al. 2015). Shortening of villi may diminish the surface area for nutrient absorption, impede the action of digestive enzymes, and lower the transport of nutrients at the villus surface (Zhang et al. 2005; Tufarelli et al. 2010). A lower VH:CD ratio could increase the turnover rate of the intestinal epithelium, resulting in a higher maintenance requirement, which can finally lead to depressed growth performance in farm animals (Van Nevel et al. 2005).

A review of the literature shows that the effect of feeding BSFLM on the intestinal morphology of chickens may vary according to the level of inclusion. The present findings are in agreement with (Dabbou et al. 2018) that diets containing up to 10% BSFLM had a negligible influence on intestinal morphology in chickens. Dabbou et al. (2018) showed that increasing the concentration of BSFLM up to 15% in the diet was detrimental to the intestinal morphology of broilers. On the contrary, feeding BSFLM as a complete replacement for soybean meal as a source of protein improved the duodenal villi height of 45-week-old laying hens but the converse for jejunum and the ileum (Cutrignelli et al. 2018).

In poultry, the microbiota predominantly occupies specific parts of the gut, including the crop, ileum, and caecum, with the caecum having the most diverse microbes (Borda-Molina et al. 2018; Kers et al. 2018). Previous studies (Song et al. 2014; Zhang et al. 2017; Awad et al. 2018) highlighted that cyclic HS modified the intestinal bacteria by increasing the *Salmonella* spp., *Escherichia coli*, and *Clostridium* spp. populations but decreasing the *Lactobacillus* spp. and *Bifidobacterium* spp. counts. In agreement with these findings, we found that the caecal *E. coli* and *Clostridium* spp. counts of the heated D0 broilers were higher than their unheated counterparts. Cao et al. (2021) suggested that heat stress can impact the composition of the gut microbiota through direct effects on body temperature or indirectly through acute or gradual changes in bird behaviour, physiological condition, intestinal integrity, and immune system activity. In the present study, incorporating 5% and 10% BSFLM in diets was beneficial in reducing the counts of caecal *E .coli* and *Clostridium* spp. in heated broilers. Dabbou et al. (2021) reported that supplementing diets with black soldier fly larvae fat lowered faecal *Clostridium* spp. and *Corynebacterium spp.* counts in broilers. Black soldier fly larvae meal is rich in medium-chain fatty acids, particularly lauric acid (Ewald et al. 2020; Kim et al. 2020). Lauric acid possesses significant antimicrobial properties and may boost animal immune response through its antimicrobial and anti-inflammatory properties in the small intestine (Devi and Kim 2014; Spranghers et al. 2017). Furthermore, the exoskeleton of black soldier fly larvae contains chitin, which acts as a prebiotic, fostering a balanced and diverse population of beneficial gut microbes and possessing immunostimulatory properties (Borrelli et al. 2017). The combined functional properties of BSFLM could potentially reduce the proliferation of harmful bacteria in the gut, improve nutrient absorption, and enhance growth performance (Fioramonti et al. 2003; Heo et al. 2013). The findings suggest that the D10 birds had higher caecal *Lactobacillus* spp., irrespective of heat treatment,

counts than the D0 and D5 broilers. Similarly, Park et al. (2017) reported that laying hens fed black solder fly pupa had increased faecal *Lactobacillus* spp. counts. Matsue et al. (2019) indicated that lauric acid had low antimicrobial activity against commensal lactic acid bacteria but strong activity against pathogenic *Bacteroides* and *Clostridium*.

Conclusion

In conclusion, the findings of the present study suggest that BSFLM can be included in heat-stressed broiler chicken diets at a level of up to 10% and still support growth performance, which is not different from the birds-fed diets with soybean meal as the source of protein. Using BSFLM as a feed ingredient may offer a potential operational strategy to enhance gut microflora and intestinal mucosal integrity and alleviate physiological stress in broiler chickens exposed to high temperatures. Currently, the use of BSFLM is receiving more attention in the Malaysian poultry feed industry as an effort to reduce dependency on imported soybean meals as a source of protein. Given that the present cost of BSFLM remains relatively high, future research should focus on investigating the potential of BSFLM as a feed supplement in mitigating heat stress and enhancing the gut health of broiler chickens in hot and humid tropical environments.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported by the Malaysian Ministry of Higher Education under the Higher Institution Centre of Excellence (HICoE) programme and the Malaysian Productivity Corporation.

ORCID

Sharmila Ahmad http://orcid.org/0000-0003-0644-684X
Hasliza Abu Hassim http://orcid.org/0000-0002-7119-6560
Suriya Kumari Ramiah http://orcid.org/0000-0002-0693-9436
Zulkifli Idrus http://orcid.org/0000-0002-2082-7430

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