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Impact of live black soldier fly larvae supplementation on laying hen performance, stress levels and excreta microbiota

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ABSTRACT

As the global demand for poultry products, particularly eggs, continues to rise, identifying environmentally sustainable alternative protein sources is crucial and insect-based feeds, such as black soldier fly larvae (BSFL) represent a promising solution. This study investigated the effects of BSFL supplementation as environmental enrichment for laying hens, assessing its impact on bird performance, integument status, excreta corticosterone metabolites (ECMs) and microbiota composition. A total of 108 Lohman Brown hens were housed in 27 cages (nine replicates per treatment, four birds per pen) and monitored between 16 and 34 weeks of age. The hens were assigned to three dietary treatments: a control group fed a commercial diet, and two experimental groups supplemented with 15 % or 30 % of live BSFL, as fed basis on the expected daily feed intake (DFI). Larvae consumption time, hens' performance and integument scores were recorded at regular intervals. Excreta samples were collected for ECMs analysis and microbiota profiling.

Hens adapted progressively to BSFL consumption, as feeding time significantly decreased over the trial, with a significant interaction between BSFL supplementation and hen age. BSFL supplementation had no adverse effect on laying performance or integument scores of the hens.

ECM concentrations were influenced by both diet and age with the BSFL-fed groups showing lower corticosterone levels than the control; particularly towards the end of the experiment. While BSFL supplementation did not significantly modify the dominant microbiota taxa, it led to structural shifts in the bacterial community, possibly influenced by age and/or the environmental factors.

Supplementing the diet of laying hens with live BSFL did not negatively influence productivity or plumage quality. While BSFL supplementation had a minimal impact on the overall microbiota diversity in the excreta, it influenced specific microbial taxa and appeared to reduce the stress level in the hens. These findings highlight the potential of BSFL as a functional feed ingredient that could enhance poultry welfare without impairing production performance.

Introduction

The poultry industry has become a leading global provider of efficient and high-quality animal protein thanks to advancements in genetics, nutrition, housing, and management strategies (Korver, 2023). However, intensive poultry farming heavily relies on soybeans as feed, which contribute to deforestation and environment degradation. To promote more sustainable farming practices, the feed sector is actively exploring alternative protein sources to reduce its reliance on soybean. Among these alternatives, insects gained recognition as a promising

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alternative feed ingredient, due to their nutritional value, cost-effective production (Sogari et al., 2023) and lower environmental footprint (Barragan-Fonseca et al., 2017).

In particular, the black soldier fly (*Hermetia illucens* L., BSF) has emerged as a highly sustainable protein source. The incorporation of live BSF larvae (BSFL) into poultry diets offers a promising way to enhance the sustainability of animal farming through a circular economy approach (Khalifah et al., 2023). In fact, compared to soybean meal, BSFL production has a significantly lower environmental impact, and it is particularly suitable for monogastric animals, as poultry (Gasco et al., 2019; Abd El-Hack et al., 2020). Additionally, BSFL supplementation was associated with improvement of animal health and welfare (Gasco et al., 2020; Bellezza Oddon et al., 2024). Currently, two main insect-based feed production methods are used: (i) processing larvae into meals or extracting their fats to obtain protein and energy to be integrated in the poultry feed, and (ii) feeding live or dehydrated whole larvae to poultry, which also serves as environmental enrichment (Fiorilla et al., 2024a; Schiavone and Castillo, 2024). While insects-based feed has been intensively studied in poultry nutrition, research on the direct use of live BSFL is relatively limited (Fiorilla et al., 2024b).

Most studies have focused on the inclusion of insect meal, emphasizing its nutritional aspects and safety (Schiavone et al., 2018, 2019; Dabbou et al., 2018; Bertinetti et al., 2019). However, feeding live BSFL to poultry feed not only provides nutritional advantages, but also improves natural behaviours, such as foraging, which are crucial for the psychophysical welfare of chickens (Biasato et al., 2022). In fact, chickens are natural foragers that consume live insects in the wild (Despins and Axtell, 1994). The inclusion of insects in their diet has been shown to mitigate harmful behaviors such as feather pecking (Tahamtani et al., 2021). Providing high-quality pecking substrates is a well-established strategy to reduce feather pecking (Monckton et al., 2020) and insect represent a particularly effective enrichment option.

Excreted corticosterone metabolites (ECMs) are widely recognized as reliable indicators of stress in birds (Möstl and Palme, 2002; Engel et al., 2022), although stress includes both detrimental conditions that affect animal fitness and adaptive responses that are crucial for managing daily challenges (Tilbrook and Ralph, 2018). Several studies have shown that environmental enrichment can influence the effects of stressors in poultry (Li et al., 2021), and ECMs often in combination with other parameters- has been widely used to assess the effects of environmental enrichment in chickens (Bongiorno et al., 2024; Bellezza Oddon et al., 2024; Bongiorno et al., 2025). This method has been validated in poultry and other avian species, offering an ethically and scientifically robust tool to monitor welfare under different environmental and management conditions (Currier et al., 2024).

The gut microbiota plays a crucial role in nutrient utilization, immune system modulation, and enhancing host health and production performance in poultry (Oakley et al., 2014; Kers et al., 2018). It consists of the microbial populations residing throughout the gastrointestinal tract and varies spatially along it due to changing in the dietary experimental groups, such as pH, oxygen, and nutrient availability. In poultry, the majority of microbial biomass resides in the ceca, where fermentation of complex carbohydrates, production of short-chain fatty acids, and modulation of the immune system occur (Liu et al., 2021a; Ali et al., 2022). Fecal microbiota, which largely reflects the microbial communities present in the ileum and caeca (Yan et al., 2019) is commonly analyzed as a practical and non-invasive proxy for gut microbial composition. Indeed, although not representing the entire gut microbiota, the excreta (in birds feces and urine, excreted through the cloaca) microbiota composition reflects both core resident populations and transient microbes, which are mainly influenced by diet, environment, and management practices and therefore can be used, for example, to discriminate the effect of diet supplementation (Yan et al., 2019). Changes in the gut microbiome of laying hens can modify productivity, feed consumption, and overall health (Yeoman et al., 2012; Kers et al., 2018; Nair et al., 2022). Diet composition is the main determinant of gut microbiota diversity and structure, with feed ingredient modifications directly affecting microbial populations (Torok et al., 2011). The supplementation of live BSFL in diets of laying hens improves growth performance, resistance to pathogens and gut microbiota composition (Star et al., 2020; Tahamtani et al., 2021; Huang et al., 2024). These effects could be due to the natural antimicrobial properties of BSFL, which contain bioactive compounds, such as lauric acid and chitin (Cattaneo et al., 2023). These components have been shown to reduce the abundance of pathogenic bacteria, such as *Escherichia coli* and *Salmonella*, thereby enhancing poultry health (Erickson et al., 2004; Lee et al., 2018).

This study aims to evaluate the effects of live BSFL as environmental enrichment for laying hens by assessing the production performance and welfare conditions in terms of plumage status, ECMs and gut microbiota composition.

Methods

The experimental trial was carried out in a commercial poultry farm located in Viterbo province (Latium region, Italy). Ethical approval was not required for the research. Nevertheless, the experimental procedures were designed following the guidelines outlined in current European and Italian legislation on animal care and use, specifically European Directive 86/609/EEC (Council European Union, 1986).

Birds, husbandry and feeding

A total of 108 Lohman Brown hens [average initial live weight (means ± standard deviation): 1320 ± 93 g] were housed from 16 to 34 weeks of age in 27 cages (9 replicates/treatment, 4 birds/cage). Each pen of each treatment was randomly assigned to one of the three experimental treatments. The cage allocation strategy aimed to ensure a balanced distribution of treatments across the entire housing environment and to control possible spatial effects. The 27 cages were then arranged in alternating order of treatments to avoid locational bias. All groups were exposed to the same environmental conditions and kept under identical housing conditions, thus minimizing differential exposure to environmental or procedural stressors.

Cages were 102 cm wide × 154 cm long × 96 cm high and the usable space for the animals was 60 cm wide × 90 cm long × 73 cm high, providing a floor area of 5400 cm^2 (1350 cm^2 per hen). Each cage was equipped with one feeder, one bell drinker, and one nest. The temperature and the relative humidity in the poultry house were set according to the Lohmann Brown-Classic Pullets/Layers Guide guidelines (20–22°C, 50–60 % relative humidity). The animals and the environmental parameters were daily checked during the whole experimental period. They were randomly divided into three dietary groups starting from weeks 16. The control group (BSFL0) was daily fed a basal crumble commercial diet. The BSFL15 group was daily fed the control diet + live BSFL calculated on the fresh matter basis as 15 % of the expected daily feed intake (DFI), equivalent to an average of 4.36, 4.48, 5.42, 5.68, and 5.11 grams of larvae (expressed on dry matter; DM) for animal for day during the trial (weeks 16-20, 20-24, 24-28, 28-32, 32-34 of age). The BSFL30 group was daily fed the control diet + live BSFL calculated on the fresh matter basis as 30 % of the expected DFI, equivalent to an average of 8.72, 8.92, 10.83, 11.32, and 10.22 grams of larvae (expressed on DM) for animal for day during the trial (weeks 16-20, 20-24, 24-28, 28-32, and 32-34 of age). The consistency of larvae chemical composition during the weeks to the trials were ensure by repeated chemical analysis of representative sample of insects. The birds' DFI was adjusted in accordance with their growth progression. The quantity of live larvae provided to each bird was modified based on the anticipated changes in DFI, resulting in a gradual increase in larvae intake over the course of the trial. The live insect larvae were given daily at 9 h 00 am. every day of the trials duration. Prior to administration, larvae were reactivated at room temperature and then placed in the feeder of each cage (Cattaneo et al., 2025). Although circadian rhythms were not explicitly considered in the experimental design, the fixed timing of daily supplementation ensured equal exposure across all treatment groups. Water was offered *ad libitum* to all birds. The chemical composition of BSFL and the experimental diets, as well as the complete experimental protocol, are described in Cattaneo et al. (2025).

Laying performance and larvae consumption time

The birds were individually labelled with a wing mark at the beginning of the experimental trial. During the experiment, mortality rates and clinical signs of illness were monitored daily. The live weight of each bird was recorded every around 30 days (namely on days 32, 56, 84, 112, and 126 of the experiment), corresponding to 20, 24, 28, 32, and 34 weeks of age for the hens. Measurements were taken using an electronic scale (Trade Shop, Portable Electronic Hunting Fishing Scale, max 40 kg, d: 5 g). In contrast, eggs were weighed with a more precise scale (Radwag WTB 2000, max 2000g, d: 0.01 g).

The average DFI on a DM basis was calculated for each cage as the total of commercial feed and BSFL consumption. Egg weight (EW) was recorded every 30 days. The Laying Rate (LR) was calculated by multiplying the Egg Production (EP; number of eggs collected daily divided by the number of hens on the same day) by 100. The Feed Conversion Ratio (FCR) was calculated as the ratio between the total average DFI to the average egg mass (obtained by multiplying the average EP by the average EW). The FCR calculation (expresses on a DM basis) included the commercial feed intake and the one derived from BSFL supplementation (where present). The average values of DFI, EP, LR, and FCR were calculated every 30 days (on days 32, 56, 84, 112, and 126 of the experiment).

The time required for each cage in the BSFL15 and BSFL30 groups to consume the larvae was measured (in minutes and decimal fractions of a minute) using a digital stopwatch (LinkDm Company). The measurement started from the consumption of the first larva and ended when all the larvae in the cage were consumed.

Welfare indicators

(i) Integument scores

On days 32, 56, 84, 112, and 126 of the experiment, all the birds were assessed for integument condition by the same operator, who scored the neck, breast, cloaca/vent, back, wings, tail, and bumblefoot lesions. A higher score indicated a better integument status. This scoring system evaluated individual body regions (scores: 1, 2, 3, or 4) as well as the overall body condition (scores: 6 to 24) (Tauson et al., 2015). The overall integument score was calculated as the sum of the scores assigned to six body regions—neck, breast, cloaca/vent, back, wings, and tail—each scored from 1 (poor condition) to 4 (intact plumage). Consequently, the total score ranged from 6 (poor overall condition) to 24 (excellent overall condition), with higher values indicating better plumage condition (Tauson et al., 2015).

(ii) Excreta corticosterone metabolites

Fresh excreta samples were collected in the morning, between 8 h 00 and 10 h 00 am, during weeks 20, 28 and 34 of age (days 32, 84, and 126 of the experiment), prior to feeding and larvae supplementation.

In each cage, fresh feces were collected immediately after defecation by direct observation with sterile tools. A plastic tray was placed under the cages to collect samples and avoid contamination. As the hens were housed in groups of four per cage and individual identification of feces was not possible, the samples were pooled by cage, resulting in a total of 27 pooled samples (one per cage). The cage was considered the experimental unit for corticosterone analysis. The collected excreta were then stored at −20°C until underwent lyophilization (Edwards MF 1000, Milan, Italy) and subsequent analyses. The methanol-based procedure described by Costa et al. (2016) was used to extract the Corticosterone Metabolites (CMs). An aliquot of each sample (0.25 g) was placed in the extraction tubes containing 3 mL of 80 % methanol (Sigma Aldrich, St. Louis, MO, USA). This mixture was thoroughly mixed for 20 min using a multivortex, sealed with a Teflon cap, and then stored at −20°C to allow the sediment to settle at the bottom. After 2 h, an aliquot (0.5 mL) of the supernatant was transferred to a new vial and evaporated at 50°C for 14 h. The dried extracts were stored in dark boxes in the freezer for 7 days before measurement.

For ECMs measurement, a multispecies ELISA kit (K014; Arbor Assays®, Ann Arbor, MI, USA) developed for various matrices, including serum, plasma, urine, dehydrated faeces, and tissue cultures, was used. An aliquot of the extract was diluted 1:10 with the assay buffer. The mixture was vortexed and allowed to rest for 5 min. The process repeated to ensure a complete steroid solubilization. Corticosterone analyses were carried out twice. The corticosterone kit (K014; Arbor Assays®, Ann Arbor, MI, USA) has the following cross-reactivity as declared by the manufacturer: 100 % with corticosterone, 12.3 % with desoxycorticosterone, 0.62 % with aldosterone, 0.38 % with cortisol, and 0.24 % with progesterone. CMs concentration was expressed in ng/g of droppings DM.

The assay's sensitivity was 11.2 ng/g for droppings. All droppings' samples were tested at multiple dilutions (1:4, 1:8, 1:16, and 1:32), and all regression slopes paralleled the standard curve (r^2: 0.985). The average recovery rate of added corticosterone to dried excreta was 97.1 %.

Characterization of the bacterial microbiota in the excreta

To characterize the composition of bacterial communities, fresh excreta samples were collected individually and then pooled for each cage (n: 27 samples per dietary group; 81 samples in total) during weeks 20, 28 and 34 of age (on days 32, 84, and 126 of the experiment). The samples were placed in sterile polyethylene bags and immediately stored at −80°C. DNA was extracted from 250 mg of sample, using the Dneasy PowerSoil Pro DNA kit (Qiagen) according to the manufacturer's protocol. Prior to the bead beating step (FastPrep-24 Classic 2500 rpm 30 s), samples were incubated at 65°C for 10 min as suggested by supplier for improving the cell lysis. After cell lysis and purification, the DNA was eluted with 100 µl of the elution buffer.

The concentration of DNA was measured using the Qubit (ThermoFisher Scientific). The amplicon polymerase chain reaction (PCR) was carried out by Biorad C1000 Touch thermal cycler in 25 µL on the following reaction mix: 12.5 ml of 2x KAPA HiFi HotStart Ready Mix, 1 µL of each primer (10 µM), extracted DNA (12.5 ng/reaction) and nuclease-free water to a final volume of 25 µL. The 16S V3-V4 region was amplified by the following primers: (i) 16S amplicon PCR FW with sequence "5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG" and (ii) primer 16S amplicon PCR REV with sequence "5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC".

The condition of PCR was 95 °C for 3 min, followed by 35 cycles - denaturing (98 °C, 30 s), annealing (55°C, 30 s) extending (72°C, 30 s) – and a final extension at 72 °C for 5 min. The PCR products (2 ml) were run on 2 % Agarose E-Gel 96 with ethidium bromide (Thermo Fisher Scientific) for size verification. Prior to the index PCR, the 16S V3 and V4 amplicons (expected size ∼550 bp) were purified from free primers and primer dimer species by a PCR clean-up step using AMPure XP beads 0.8X (Beckman Coulter). Index PCR attaches dual indices and Illumina sequencing adapters. It was performed in a 50-µl reaction volume containing 25 µl of 2 × KAPA HiFi HotStart ReadyMix, 5 µl of each forward and reverse index primer (Nextera XT Index Kit v2, Illumina), and 5 µl of the purified PCR products. Second PCR clean-up step used AMPure XP beads (Beckman Coulter) to clean up the final library before quantification. The indexed PCR products were then purified with 56 µl of AMPure XP and quantified using Qubit (ThermoFisher Scientific).

Before sequencing, libraries with unique indexes were pooled; pools were quantified using Qubit and checked for quality on Bioanalyzer DNA 7500 chip (V3 and V4 primer pairs in the protocol yield a final library of ~630 bp); DNA concentration in nM was calculated based on DNA amplicon size. Pooled libraries are denatured, diluted and sequenced by the MiSeq platform (Illumina) using Miseq reagent kit v2 (sequencing configuration: 2 × 250 bp PE). To enhance the performance and accuracy of the sequencing process 10 % PhiX was spiked in. For metagenomic samples, >150,000 reads/sample were obtained.

Statistical analysis and bioinformatics

(i) Laying performance, larvae consumption time and welfare indicators

Statistical analyses on performances, larvae consumption time, integument scores and ECMs parameters were carried out with R software, version 2023.03.1 (R Development Core Team, 2011). Dietary supplementation with BSFL, as well as hens' age (measured in weeks) were treated as independent variables and designated as model covariates. Their values were assumed to be independent of other variables included in the study. Multiple regression models were employed to analyze various response (dependent) variables, including egg weight, laying rate, and others, to evaluate the effects of the independent variables and their interaction. To account for the dependency structure among observations from hens housed within the same cage, cage number was included as a random effect in the models.

A Linear Mixed Effects models was applied for each response variable concerning all the parameters, considering the effects of diet, time and their interaction. The "nlme" package (Pinheiro et al., 2021) was applied, together with the package "emmeans" (Lenth, 2023) for a multivariate comparison of the covariates in a post hoc analysis. Specifically, the sets of means estimated for each group of the covariate of interest, a set due to the interaction effect of the second covariate, were compared using the Mahalanobis distance and Hotelling's T statistic (Hotelling, 1931). The root-mean-squared error (RMSE) was computed for evaluating model accuracy. Differences among the means with $P \leq 0.05$ were accepted as statistically significant.

(ii) Microbiota of the excreta

The GAIA platform was used for quality checking and trimming the metagenomic reads (Paytuví et al., 2019). BBDuk tool within the GAIA platform was used for removing adapter sequences and low-quality portions of reads. Burrows-Wheeler Aligner (Li and Durbin, 2010) was used to map high-quality reads to a custom-made database created from NCBI sequences (NCBI Resource Coordinators, 2016). The reads were classified into the various taxonomic levels with an in-house lowest common ancestor algorithm. Rarefaction curves were generated to evaluate sequencing depth, and the taxonomic composition was analysed (R software). Minimum identity thresholds were applied to classify sample reads into genus, family, order, class, phylum and domain levels. Alpha and beta diversities were calculated using "mia" package (McMurdie and Holmes, 2013). Alpha-diversity was assessed using taxa richness and the Shannon Index, while beta-diversity was evaluated using Principal Coordinates Analysis (PCoA), based on Bray-Curtis distance values to explore the dissimilarity in taxonomic composition between samples. Principal Component Analysis (PCA) was performed to identify patterns and trends within the data. Euclidean distances were calculated and centered log-ratio (CLR) values were employed to normalise the relative abundance data of microbial communities, ensuring suitability for statistical analysis. DESeq2 was used to perform differential abundance analysis to identify microbial taxa that are significantly different in abundance between different conditions or groups (Love et al., 2014).

The model for this analysis considered time (age of 20, 28, and 34 weeks), BSFL supplementation level (BSFL0, BSFL15 BSFL30) and their interaction. BSFL supplementation was treated as an independent variable to assess its interactions with the other factors. Results were considered statistically significant if the adjusted P-value (False Discovery Rate, FDR) was less than 0.05 and the Log Fold Change (LFC) exceeded ±1.

Additionally, to assess the differences in the relative abundance of the three dominant phyla over time and between dietary treatments, non-parametric Kruskal–Wallis tests were applied.

Results

Laying performance

The dietary supplementation of live BSFL to laying hens did not significantly affect the performance parameters of the laying hens ($P >$ 0.05; Table 1). In contrast, age had a significant impact on all measured parameters ($P < 0.001$). Notably, there was an interaction effect between larvae supplementation and hen age on egg weight ($P = 0.013$, Supplementary Fig. A). Egg weight gradually increased from 51.9 g at 20 weeks to 57.6 g at 28 weeks ($P < 0.001$) before slightly decreasing to 55.6 at 32 weeks and 56.2 g at 34 weeks of age, respectively. Specifically, egg weight increased with age in all groups but the trend was more pronounced in BSFL30 from 32 weeks onwards. Hens' weight increased significantly from 20 to 34 weeks of age ($P < 0.001$). Over the weeks, the laying rate significantly increased from 35.4 % at 20 weeks to a peak of 90.9 % at 32 weeks, before slightly decreasing to 85.9 % at 34 weeks of age ($P < 0.001$). DFI also increased significantly with age, from 415.9 g/day/cage at 20 weeks to 644.2 g/day/cage at 34 weeks of age ($P < 0.001$). Additionally, FCR improved significantly with age, decreasing from 5.50 at 20 weeks to 2.63 at 24 weeks, and then slightly increasing to 3.65 at 34 weeks of age ($P < 0.001$).

Larvae consumption time

The laying hens showed significant differences in larval consumption time between the two supplemented dietary groups (6.02 *vs.* 12.13 min, $P < 0.001$, Table 2) when aggregating data across the entire analysis period, becoming progressively faster during the experimental trial ($P <$ 0.001). Interestingly, an interaction effect was observed between larvae supplementation and hen age on consumption time ($P = 0.02$; Table 2 and Supplementary Fig. B). Specifically, in the BSFL 15 group, larvae consumption time showed a similar trend at 20 and 24 weeks, followed by a sharp decrease between 24 and 28 weeks, after which it stabilized. Conversely, in the BSFL30 group, consumption time remained similar between 24 and 28 weeks but decreased steadily from 32 weeks onwards.

Between 20 and 24 weeks of age, the birds required an average of 12 min to acclimatize to the larvae consumption, then the hens reduced their consumption time around 10 min. Towards the end of the trial, the consumption time stabilized at 5.30–6.00 min.

Welfare indicators

(i) Integument scores

BFSL supplementation had no significant effect on the integument scores of the laying hens ($P > 0.05$) (Table 3). However, hen age significantly influenced this behavioural parameter ($P < 0.001$). Between weeks 20 and 24, a progressive decrease in integument scores was observed, reaching the lowest point at week 28. After this period, plumage condition improved, returning to levels comparable to those recorded in the first weeks of the trial.

Table 1
Laying performance of the laying hens depending on BSFL supplementation (BSFL), weeks of age (T) and their interactions (BSFLxT).

Items	BSFL supplementation (BSF)			Weeks of age (T)				RMSE	P		
	BSFL0	BSFL15	BSFL30	20	24	28	32	34	BSFL	T	BSFL × T
Hens weight, kg	1.77	1.76	1.85	1.66^{B}	1.74^{B}	1.87^{A}	1.83^{A}	1.86^{A}	0.162	0.057	<.001	0.0698
Laying rate, %	74.0	79.8	77.4	35.4^{C}	82.6^{B}	90.7^{A}	90.9^{A}	$85.9^{A,B}$	6.764	0.344	<.001	0.548
Egg Weight, g	54.2	56.2	56.4	51.9^{E}	56.1^{C}	57.6^{A}	55.6^{D}	56.2^{B}	3.817	0.108	<.001	0.013
DFI /cage (g/d) + larvae	540.5	539.8	564.8	415.9^{E}	473.9^{D}	598.2^{C}	609.5^{B}	644.2^{A}	66.416	0.770	<.001	1.000
FCR /cage (g/g, DM basis) + larvae	3.78	3.41	3.69	5.50^{A}	2.63^{B}	3.07^{B}	3.30^{B}	$3.65^{B,C}$	0.815	0.467	<.001	0.983

BSFL0: control diet; BSFL15: control diet + 15 % live black soldier fly larvae; BSFL30: control diet + 30 % live black soldier fly larvae; RMSE: root-mean-square error. A-E Values with different superscript letters within the same line and effect are significantly different ($P < 0.01$).

Table 2
Larvae consumption time by the laying hens depending on BSFL supplementation (BSFL), weeks of age (T) and their interactions (BSFL × T).

	BSFL supplementation (BSFL)		Weeks of age (T)					RMSE	P		
	BSFL15	BSFL30	20	24	28	32	34		BSFL	T	BSFL × T
Consumption time (minutes. seconds)	6.02^{B}	12.13^{A}	11.25^{A}	12.21^{A}	10.23^{A}	5.37^{B}	6.00^{B}	2.582	<0.001	<0.001	0.02

BSFL0: control diet; BSFL15: control diet + 15 % live Black soldier fly larvae; BSFL30: control diet + 30 % live Black soldier fly larvae; RMSE root-mean-square error. A-B Values with different superscript letters within the same line and effect are significantly different ($P < 0.01$).

Table 3
Integument scores of the laying hens depending on BSFL supplementation (BSFL), weeks of age (T) and their interactions (BSFL × T).

	BSFL supplementation (BSFL)			Weeks of age (T)					RMSE	P		
	BSFL0	BSFL15	BSFL30	20	24	28	32	34		BSFL	T	BSFL × T
Total scores	23.31	23.45	23.57	23.88^{A}	23.49^{B}	22.63^{C}	23.57^{B}	$23.63^{A,B}$	0.692	0.106	<0.001	0.389

BSFL0: control diet; BSFL15: control diet + 15 % live black soldier fly larvae; BSFL30: control diet + 30 % live black soldier fly larvae; RMSE: root mean-square error. A-B Values with different superscript letters within the same line and effect are significantly different ($P < 0.01$).

(ii) Excreta corticosterone metabolites

Excreta corticosterone metabolites (ECMs) of the laying hens (Table 4) were influenced by both diet and time ($P < 0.001$ and $P = 0.001$, respectively). In particular, live BSFL administration groups displayed lower corticosterone concentrations than the control group ($P < 0.001$). Independently of the administration of live BSFL, the ECMs significantly decreased throughout the trial, with the most notable decline occurring between 28 and 34 weeks of age. This trend suggests that corticosterone concentrations tended to decrease as the trial progressed.

Characterization of the bacterial microbiota in the excreta

The average quantity of DNA extracted from the samples was 80 ng/µl. In the sequencing >150,000 reads/sample were obtained. 8, 32, 21, and 39 % of samples resulted in less than 10 ng/ml, from 10 to 50 ng/ml, from 50 to 10 ng/ml and from 100 to 240 ng/ml, respectively. The pre-processing filtered out species that were not present in at least two samples with a minimum of two counts each. The data were successfully normalized to a range of counts between 83,538 and 289,219. The rarefaction curves indicate that the sequencing depth is of sufficient quality to capture the diversity of the tested diets (Supplementary Fig. C).

Across all diet supplements and age, the most predominant bacteria phyla in the excreta were Firmicutes, Proteobacteria and Bacteroidetes (Fig. 1). Firmicutes decreased significantly from week 20 to 34, while Proteobacteria increased. On the contrary Bacteroidetes increased from week 20 to 28 and then decreased from week 28 to 34, reaching back to the initial relative abundance. Similar behaviour is noticed in all dietary groups (Fig. 1).

The dominant genera appear to be dependent on the age of the hens: Lactobacillus, Weissella and Enterococcus are dominant at week 20, Lactobacillus, Enterococcus, Bacteroides, Gallicola, Weissella at week 28 and Gallicola, Enterococcus, Kurthia, Ignatzschineria, Acinetobacter, Lactobacillus, Clostridium at week 34. At each time, the supplementation with live BSFL had no relevant impact on the dominant genera (Fig. 2). The richness values have a widespread, with a concentration of samples around the middle of the range (~200–250) in week 20. In week 28, they are more narrowly distributed, clustering closer to ~250 and in week 34 the spread is wider again, similar to T1, but with a slight shift toward higher richness values (~250–300). There is a highly increase in richness from week 20 to 28, while from week 28 to 34 there are no significant differences (Fig. 3, left). The richness values across the dietary experimental groups range between 100 and 300, with no significant differences between the groups ($P > 0.05$; Fig. 3, right).

Regarding the Shannon index week 20 has a relatively compact distribution, suggesting less variation in alpha diversity, while week 28

Table 4
Excreta corticosterone metabolites (ECM) (ng/g) concentration of laying hens depending on BSFL supplementation (BSFL), weeks of age (T) and their interactions (BSFL × T).

	BSFL supplementation (BSFL)			Weeks of age (T)					RMSE	P		
	BSFL0	BSFL15	BSFL30	20	24	28	32	34		BSFL	T	BSFL × T
Excreta corticosterone metabolites (ng/g)	52.89^{A}	36.54^{B}	37.52^{B}	$44.11^{A,B}$	45.97^{A}	$40.79^{A,C}$	$40.02^{B,C}$	40.70^{C}	5.328	<0.001	0.001	0.058

BSFL0: control diet; BSFL15: control diet + 15 % live black soldier fly larvae; BSFL30: control diet + 30 % live black soldier fly larvae; RMSE: root-mean-square error. A-C Values with different superscript letters within the same line and effect are significantly different ($P < 0.01$).

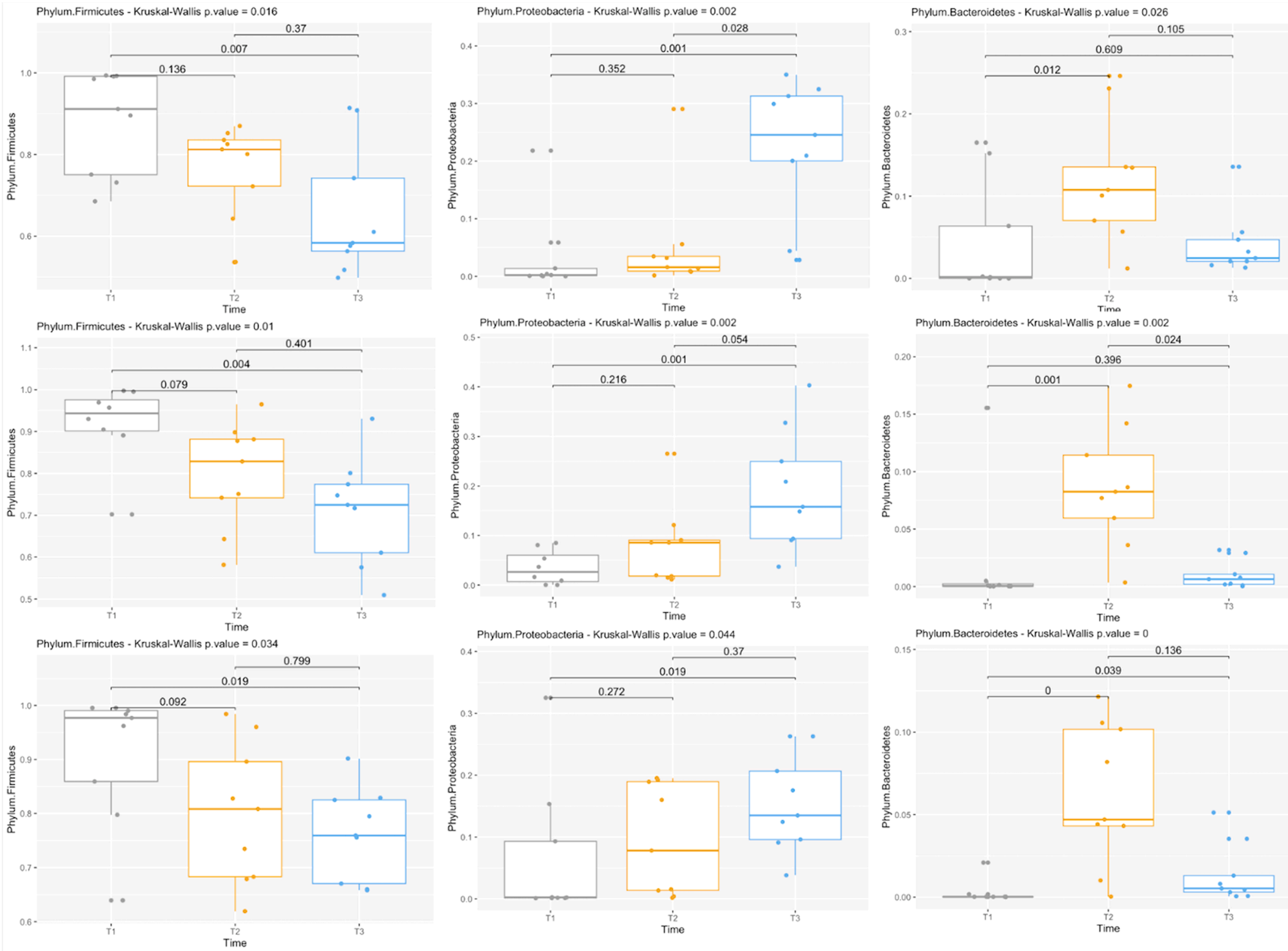

Fig. 1. Kruskal-Wallis of the three dominant Phyla of bacterial populations in the excreta of hens across different time points (T1: week 20, T2: week 28, T3: week 34) and dietary supplementation levels with black soldier fly larvae (BSFL0: Control diet; top, BSFL15: control diet + 15 % live black soldier fly larvae; middle, BSFL30: control diet + 30 % live black soldier fly larvae; bottom). Each dot represents an individua sample (cage). Y-axis indicate the relative abundance (1: 100 %). *P*-values above the violins were obtained using Kruskal–Wallis tests to compare richness between groups.

shows a broader distribution, indicating greater variability. Week 34 is relatively narrow with slightly higher diversity values compared to week 20. There is significant difference between week 20 and 28 and between week 20 and 34, while the difference between week 28 and 34 is lower (Fig. 4, left). The three dietary groups (BSFL0, BSFL15, BSFL30) have relatively similar and symmetric shapes (Fig. 4, left), indicating comparable diversity distributions and no significant skewness. There are no significant differences in the Shannon index between the dietary treatments ($P > 0.05$; Fig. 4, right).

Beta diversity among bacterial samples, based on the Bray-Curtis distance metric, analysed with PCoA (Fig. 5, left) indicate that the first two axes explain a total of 51.7 % of the variation (40.9 % by PCoA1 and 10.8 % by PCoA2). There is a progression of T1, T2, T3 samples along PCoA1 and PCoA2, indicating shifts in microbiota composition, for example week 20 samples cluster distinctly from weeks 28 and 34, showing that bacterial composition changes significantly over time, despite the diet supplement. Although no distinct cluster can be identified for the BSFL supplementation, the BSFL supplemented samples tend to cluster within each level of supplementation while control is more scattered. In the PCA (Fig. 5, right) bacterial communities vary across different dietary treatments and age of hens. The first principal component (Dim1) explains 31.1 % of the variance in bacterial community composition, while the second (Dim2) explains 16.3 %. Together, these two axes account for nearly half (47.4 %) of the total variation. Samples with similar treatments (dietary supplementation levels and time points) tend to group together, indicating that the microbiota composition within each group is similar. Control groups (week 20-BSFL0, week 28-BSFL0, week 34-BSFL0) are less distinct from BSFL-supplemented groups, and within BSFL-supplemented dietary groups, the degree of clustering differs between supplementation levels (BSFL15 and BSFL30) and time. Regarding the individual contribution genus Jeotgalibaca has a strong directional influence along Dim1, indicating that it contributes significantly to the differences observed between groups. Genera Gallicola and Atopostipes also contribute meaningfully to the variation, suggesting responses to the diet and/or temporal changes. The placement of vectors in relation to sample clusters suggests that these genera are more abundant or prevalent in specific groups.

Concerning the differential abundance, the P-values resulting from the analysis were adjusted for multiple tests to control for false positives. The differential abundance analysis identifies the significant difference in terms of taxa comparing diets and age. Several genera significantly change concentrations in the excreta during the experiment following BSFL diet supplementation: most taxa increased with BSFL supplementation and very few decreased (Fig. 6; Supplementary Table A).

In particular, Actinomyces and Klebsiella consistently increased with both BSFL supplementation at all sampling points (weeks 20, 28 and 34). The genera *Miniimonas, Georgenia, Morganella*, and *Urmitella* showed significant increases with both BSFL supplementations, except at weeks 28. At week 20, *Leucobacter, Sedimentibacter, Proteus, Citrobacter*, and

Fig. 2. Relative abundance of the bacterial genera in the excreta of hens across different time points (T1: week 20, T2: week 28, T3: week 34; top, middle, bottom) and dietary supplementation levels with black soldier fly larvae (BSFL) (BSFL0: control diet; BSFL15: control diet + 15 % live black soldier fly larvae; BSFL30: control diet + 30 % live black soldier fly larvae).

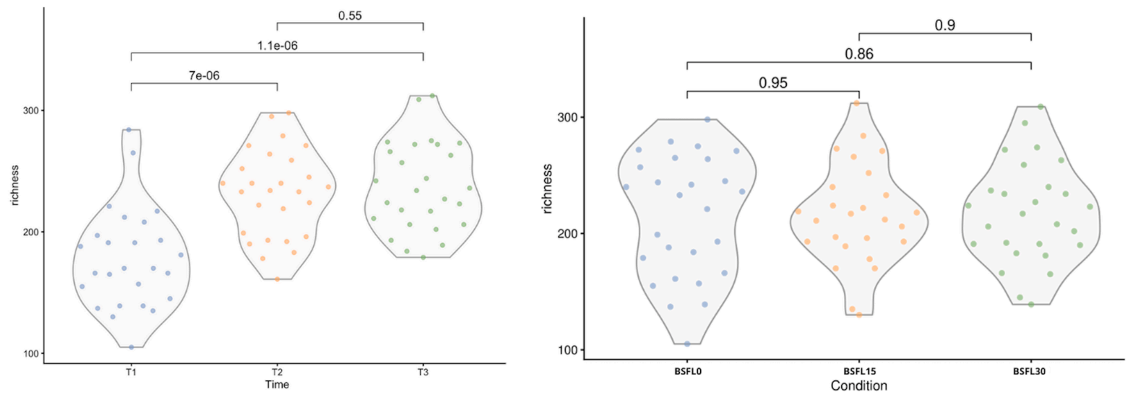

Fig. 3. Violin plots of the richness of bacterial populations in excreta of hens across different time points (T1: week 20, T2: week 28, T3: week 34; left) and dietary supplementation levels with black soldier fly larvae (BSFL) (BSFL0: control diet; BSFL15: control diet + 15 % live black soldier fly larvae; BSFL30*:* control diet + 30 % live black soldier fly larvae; right). Each dot represents the richness value for an individual sample (cage). The P-values above the violins indicate statistical comparisons of richness between the groups.

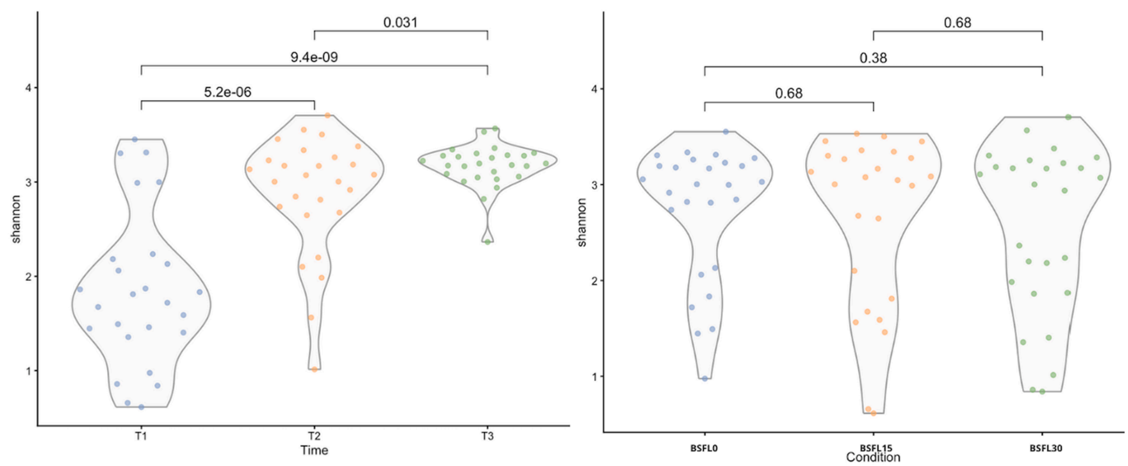

Fig. 4. Violin plots of the Shannon index of bacterial populations in excreta of hens across different time points (T1: week 20, T2: week 28, T3: week 34; left) and dietary supplementation levels with black soldier fly larvae (BSFL) (right; BSFL0: control diet; BSFL15: control diet + 15 % live black soldier fly larvae; BSFL30*:* control diet + 30 % live black soldier fly larvae). Each dot represents the Shannon index value for an individual sample (cage). The P-values above the violins indicate statistical comparisons of Shannon diversity index between the groups.

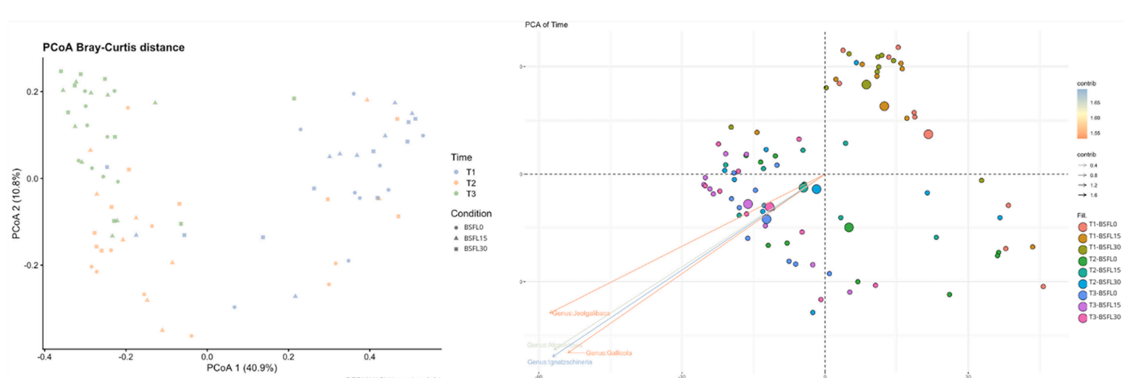

Fig. 5. Principal Coordinate Analysis plots (left) and Principal Component Analysis (right) of bacterial populations in excreta of hens across different time points (T1: week 20, T2: week 28, T3: week 34; left) and dietary supplementation levels with Black soldier fly larvae (BSFL) (right; BSFL0: control diet; BSFL15: control diet + 15 % live black soldier fly larvae; BSFL30*:* control diet + 30 % live black soldier fly larvae). Each dot represents an individual sample (cage). The contribution of individual bacterial genera is indicated by vectors (arrows).

Anaerovorax all increased with both BSFL supplementations, while *Intestinimonas* decreased. Among these, Citrobacter decreased following the 15 % BSFL supplementation, but only at week 28. At week 28 only *Fusobacterium*,which also increased at week 34, and *Oblitimonas* increased with both BSFL supplementations, while Brachybacterium showed a decrease. At week 34, the genera that consistently increased

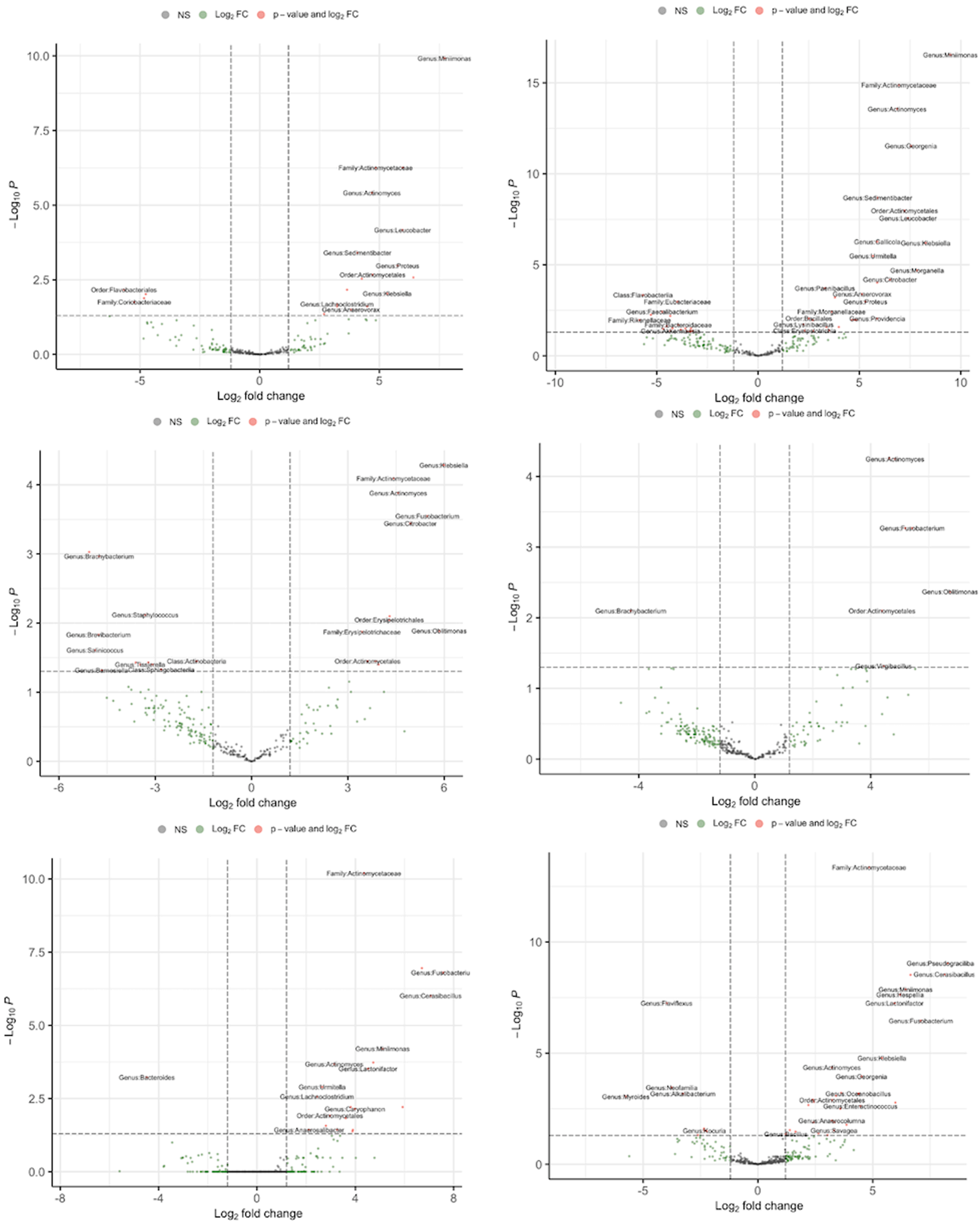

Fig. 6. Volcano plots of the comparison of bacterial populations in the excreta of hens across different time points (T1: week 20, T2: week 28, T3: week 34; left) and dietary supplementation levels with black soldier fly larvae (BSFL) (right; BSFL0: control diet; BSFL15: control diet + 15 % live black soldier fly larvae; BSFL30: control diet + 30 % live black soldier fly larvae). X-axis indicates the difference in concentration of bacteria between the two groups: BSFL15 (left) or BSFL30 (right) compared to BSFL0 at the different time points T1 (top), T2 (middle), T3 (bottom). Positive and negative values indicate increase and decrease in concentration, respectively. Y-axis indicates the statistical significance of the differences (P-value). Each dot represents a single taxon (e.g., a genus or an order).

with both BSFL supplementations included *Pseudogracilibacillus, Cerasibacillus, Hespellia, Lactonifactor, Proteiniborus, Salmonella*, and *Lachnoclostridium.*

Discussion

Laying performance

The results we obtained provide evidence that the provision of live BSFL, when fed alongside a standard diet, does not impair the laying performance of hens. Our results align with those reported by Cattaneo et al. (2025). Star et al. (2020), who showed that laying rate, egg weight, and egg mass parameters of laying hens were not affected by the provision of live BSFL on top to a soy-free diet. The authors also observed lower DFI and lower FCR in hens fed live BSFL (Star et al., 2020). Similarly, Bellezza Oddon et al. (2024) found that feeding 6 % live BSFL to two native dual-purpose chicken breeds, Bionda Piemontese and Bianca di Saluzzo, decreased DFI compared to the control group during the whole experiment. However, they found no differences in FCR between the control and BSFL groups in these breeds. Tahamtani et al. (2021) found that hens given *ad libitum* access to live BSFL consumed less concentrate and gained more weight. In addition, the 10 % live BSFL exhibited steeper growth curves compared to the 20 % live BSFL group. Lokaewmanee et al. (2023) showed that the addition of 10 g/kg live BSFL decreased feed intake and FCR of laying hens, contrary to 20 and 30 g/kg live BSFL. Furthermore, the daily provision of live BSFL (10 % of the expected DFI) to turkeys resulted in higher DFI and body weight gain, along with a lower FCR compared to the control group (Veldkamp and van Niekerk, 2019). The results of our research are in line with those reported by Bongiorno et al. (2024) for Label Necked Neck chickens fed live BSFL at 10 % of the expected DFI, where live weight, DFI and FCR were unaffected. Similarly, Gariglio et al. (2023) showed that the growth performance of Muscovy ducks was not affected by the provision of 5 % of live BSFL and Yellow mealworms. Similarly, Fiorilla et al. (2024a) studying slow-growing chickens supplemented with 5 % expected DFI of whole dehydrated BSFL or whole live BSFL, from 39 to 174 days of age, detected no change in FCR comparing to the different dietary treatment.

These differences are probably related to the lower amounts of larvae provided to the studies compared to our research (5 % and 10 % *vs.* 15 and 30 % of expected DFI of live larvae).

The age of the hens had a significant impact on their productive performance. As the hens aged, their DFI increased, reflecting their growing energy and nutrient requirements for maintenance and egg production. This trend is consistent with the natural progression of feed intake as hens mature and reach their peak laying period. Similarly, the high FCR detected at the beginning of the trial (week 20) can be related to the lower productivity detected in this period, as still the beginning of the oviposition period (Tůmová et al., 2014). The significant decrease in FCR with age suggests improved feed utilization efficiency, particularly at 24 weeks.

Although the intake of dry matter from the larvae was relatively low, the volume of larvae ingested may have initially influenced intestinal transit time, indirectly affecting DFI (Bellezza Oddon et al., 2024). However, as the hens' gastrointestinal systems adapted, this effect diminished. These findings suggest that the hens' development and production needs primarily drive the observed increase in DFI with age, while the high nutritional quality of BSFL supports overall health and welfare.

The consistent feeding patterns across different dietary treatments further highlight the importance of age and physiological adaptation in influencing feed intake and efficiency in laying hens. Bovera et al. (2014) reported that, in their study of Lohmann Brown laying hens from 20 to 36 weeks of age, FCR decreased, with improvements in laying rate and body weight gain.

The egg weight progressively increased over the course of the experimental trial, as physiologically observed during the normal production cycle (Travel et al., 2011). The significant interaction between BSFL supplementation and hens age on egg weight may reflect an optimal adaptation of the animals to this new ingredient, as well as age-related metabolic changes.

Larvae consumption time

Laying hens initially required an adaptation period to consume BSFL when introduced as a new feed ingredient, requiring more time to complete larvae consumption during the first weeks of the trial compared to the following weeks. This demonstrates the hens' inherent ability to adapt to new stimuli in their environments, as suggested by Fiorilla et al. (2024a). Bellezza Oddon et al. (2024) found that when two autochthonous dual-purpose chicken breeds, Bionda Piemontese and Bianca di Saluzzo, were fed BSFL at 6 % of the expected DFI, the time required for larva consumption decreased over the course of the trial. Tahamtani et al. (2021) showed that hens in the 10 and 20 % treatment groups consumed their entire portion of live larvae in approximately 5 min. However, when provided *ad libitum*, laying hens spaced out their larvae consumption throughout the day, likely due to the constant availability, which decreased the perceived value of the larvae (Tahamtani et al., 2021). The same authors also reported significant individual variation in larvae consumption, suggesting that not all hens consumed an equal amount, which is important to consider in groups where competition and rank order can influence individual intake (Shimmura et al., 2008). This variability may have contributed to the differences in response within treatment groups in our study, as the supplementation was administered at the cage level. Future studies could benefit from individualised measurement of insect intake, such as through video analysis, sensor-based tracking, or controlled individual feeding systems, to better measure the intake and reduce variation within groups. Anyway this reflect a natural behaviours of the animals and so represent a natural occurring-difference. Bellezza Oddon et al. (2021) also reported similar findings in broilers fed live BSFL and yellow mealworm (*Tenebrio molitor* L.) at 5 % of the expected DFI. The broilers took more time to consume this new ingredient during the first week compared to the following weeks. Similarly, Gariglio et al. (2023) demonstrated that Muscovy ducklings needed longer times to consume larvae at 7–11 days of age, averaging approximately 544 s, whereas by 21–25 days of age, this time had decreased to 125.7 s. In contrast, research by Veldkamp and van Niekerk (2019) on turkeys showed that BSFL provided at 10 % of the expected DFI was consumed extremely quickly, within 2 min, with no adaptation period required, even at the beginning of the study. These differences in larva consumption time are likely due to the species-specific feeding behaviors and patterns.

Welfare indicators

(i) Integument scores

A total score $\geq$ 18–20 usually indicates good feather cover (Tauson et al., 2015), indicating that the hens already had a satisfactory plumage condition. In our trial, the administration of live BSFL did not improve the plumage status of the laying hens. This lies in contrast with the findings of Star et al. (2020), who observed a reduction in plumage damage in older laying hens after the provision of live BSFL over a 12-weeks period. Bellezza Oddon et al. (2024) showed an improvement in plumage status, with higher total integument scores identified in Bionda Piemontese chicken breeds fed 6 % of live BSFL compared to the control group. Veldkamp and van Niekerk (2019) registered lower feather pecking incidents, particularly on the back and tail base, when BSFL were added to the diet of young turkey poults. Since poultry infections can occur by oral ingestion of dust contaminated by airborne movement during feeding or pecking (Namata et al., 2008; Becher et al., 2012), maintaining an optimal plumage status over time, as observed in the present research, is a fundamental aspect for successful rearing.

(ii) Excreta corticosterone metabolites

The assessment of stress responses in animals is crucial for evaluating their welfare, as stress can significantly impact on immune functions and behaviour (Weimer et al., 2018). Corticosterone, a hormone produced by the hypothalamus-pituitary-adrenocortical cascade in response to stressful stimuli such as human presence, fear of capture, and handling stress, may be employed as an indicator of stress status, in combination with other parameters like behavioural observations, physical condition, and gut microbiota (Rettenbacher et al., 2004; Wolf et al., 2024). Among the various methods, excreta corticosterone analysis is a well-established, non-invasive, and non-disruptive approach, allowing for stress-free sample collection. This method quantifies stress-induced alterations in daily corticoid rhythms (Carere et al., 2003; Alm et al., 2014), while preserving normal animal behaviour (Hirschenhauser et al., 2012). The measurement of corticosterone in faeces provides valuable insights into the physiological state of the animal, particularly during periods when blood samples are not available (Kouwenberg et al., 2015). Stress responses in birds are complex and multifactorial, involving neuroendocrine, behavioural and environmental interactions. Although corticosterone concentration alone is not sufficient to determine the overall welfare status of chickens (Jimeno et al., 2018), as it is a metabolite whose levels can be influenced by a variety of physiological and environmental factors, it should not be interpreted as a direct indicator of stress. Nevertheless, when interpreted in conjunction with other welfare indicators, corticosterone can provide valuable insights into the physiological state of birds (Wolf et al., 2024). Higher corticosterone concentrations have been associated with caged hens (Franciosini et al., 2005), which spend more time dustbathing and scratching (Pavlík et al., 2008). The study of Bellezza Oddon et al. (2024) demonstrated that while live BSFL positively influenced hen behavior and plumage, no differences in ECMs levels were observed between groups.

On the contrary, the decreasing trend observed in our study offers interesting insight into the positive relation between live insect supplementation, larvae behaviour, and welfare status. Furthermore, Alm et al. (2014) revealed that several factors, such as age, sex, diet, metabolic rate, social status, early life experience, diurnal and seasonal variations, and individual differences in hormone metabolism should be considered when interpreting corticosterone data. Interestingly, Dawkins et al. (2004) compared three non-invasive welfare measures (egg-shell quality, excreta corticosteroid, and behavioural preferences) in 32 ISA Brown hens at 15 weeks of age, from day 1 to day 5. Over time, the three measures did not follow the same pattern; for instance, excreta corticosterone decreased in all the groups on day 4, while egg-shell measurements declined on day 3 and then increased on day 5. Therefore, the relevance of considering these parameters over time through careful analysis and combining more evaluations among them is evident to obtain robust and replicable outcomes. Moreover, it is relevant not to rely on just one moment but to consider how that indicator changes with time. Consistent with this perspective, our study showed a time-dependent decrease in ECM concentrations and, in parallel, a significant reduction in corticosterone levels in the BSFL-supplemented groups compared to the control groups. These results may reflect an adaptation process to the experimental conditions and, in the case of the two experimental groups, to the introduction of an external stimulus such as live larvae in the diet. Finally, both the age and diet of the hens had a discernible effect on the corticosterone concentration in the excreta, which at the same time explains the importance of the interaction between BSFL supplementation and time on the data. In conclusion, while the reduced hormone levels suggest improved hen welfare with the introduction of live larvae, a comprehensive assessment using additional parameters and further research is required to support this interpretation.

Characterization of the bacterial microbiota in the excreta

In agreement with previous studies on faecal sample of hens (Videnska et al., 2014a), we found that *Firmicutes, Bacteroidetes and Proteobacteria* were the major phyla in all the excreta samples. The trend in the relative abundance of main phyla, is mainly associated with the age of hens and might be influenced with the BSFL supplementation.

More specifically, the decrease in *Firmicutes* from week 20 to 34 is consistent with studies showing that *Firmicutes* often dominate the gut microbiota in younger hens, but decrease as hens age (Videnska et al., 2014b; Han et al., 2019). This phylum includes many beneficial bacteria involved in the fermentation of dietary fibers and production of short-chain fatty acids, which are crucial for gut health (Liu et al., 2021a; Ali et al., 2022). On the other hand, the Proteobacteria, which include many opportunistic pathogens, tend to increase with age (Joat et al., 2021). The fluctuation in *Bacteroidetes*, increasing initially and then decreasing, could be due to BSFL dietary supplementation. In fact, diet and age can influence the abundance of *Bacteroidetes*, which are known for their ability to degrade complex carbohydrates (Oladele et al., 2024). Videnska et al. (2014b) observed an increase in *Firmicutes* accompanied by a decline in *Bacteroidetes* in laying hens from two to six months of age. Seven months old animals and more, on the contrary, were characterized by a constant ratio of *Bacteroidetes* and *Firmicutes*. To date, is still unknown how caecal microbiota can perceive the age and body weight of the birds.

Regarding the genera, *Lactobacillus*, known for its beneficial role in the gut microbiota, because it contributes to the production of lactic acid and maintains gut health, dominates all ages, though fluctuating over time (Khan and Chousalkar, 2021). Maintaining gut health is not only important for physiological function, but is also recognized as a key indicator of animal welfare. *Weissella* is often associated with the fermentation process and has been found in various poultry studies to be part of the gut microbiota (Yan et al., 2019). *Enterococcus* is known for its resilience and ability to thrive in various environments, including the intestine of mammals (Krawczyk et al., 2021). Its consistent presence across different ages confirms previous studies on its capability to survive in the excreta microbiota of hens (Bertelloni et al., 2015). *Bacteroides, Gallicola, Kurthia, Ignatzschineria, Acinetobacter* and *Clostridium* appear only with aging of the hens, as already documented in young pullets from week 3 to week 16 (Liu et al., 2021b). All these genera are associated with various functions, both positive and negative on animal health. For example, *Bacteroides* is crucial for the digestion of complex carbohydrates and maintaining gut health (Fan et al., 2023). *Clostridium* is known for its diverse roles, including both beneficial and pathogenic species (Guo et al., 2020). *Gallicola, Kurthia* and *Ignatzschineria* are less commonly reported in birds and their role in the gut microbiota should be better characterised. In this study, the supplementation with live BSFL had no significant impact on the dominant genera, suggesting that the dominant core microbiota in the excreta remained stable despite dietary changes (Martínez et al., 2013; Zoelzer et al., 2021).

Bacterial richness increased significantly from week 20 to 28 and remains elevated at week 34 compared to week 20, suggesting a plateauing or stabilization of diversity levels after week 28. Shannon Index also increases from week 20 to 28, indicating a larger bacterial diversity at week 28, then slightly decreases at week 34 but still remains higher than week 20. The significant differences indicate that at the three time points the bacterial community has a different structure, possibly influenced by age and/or the environment. Regarding the dietary supplements (BSFL15 and BSFL30), they did not significantly alter bacterial alpha diversity compared to the control group, thus suggesting that the BSFL supplements did not disrupt the richness and evenness of bacterial taxa in the present study. The observed increase in richness from week 20 to 28, followed by stabilization from week 28 to 34, is consistent with studies showing that increase microbial diversity by aging (Joat et al., 2021). The lack of significant differences in richness between dietary groups suggests that BSFL supplementation does not drastically alter the overall richness of the gut microbiota (Ndotono et al., 2022b), which aligns with findings that BSFL can be integrated into poultry diets without disrupting microbial balance (Ndotono et al., 2022a). The results obtained with the PCoA and PCA analysis confirm significant shifts in bacterial microbiota composition over time. The lack of distinct clusters for BSFL supplementation in PCoA, but the tendency for BSFL samples to cluster within each level suggests that while BSFL does not drastically alter the overall microbiota composition, it rather provides a stabilizing effect. This is in line with previous studies showing that BSFL can be integrated into poultry diets without causing significant disruptions to gut microbiota (Biasato et al., 2020; Zhao et al., 2023). The contributions of *Ignatzschineria, Jeotgalibaca, Gallicola*, and *Atopostipes* to the observed variation indicate their potential roles in adapting to dietary and temporal changes. For example, the genus *Ignatzschineria* is most found in the digestive tract of insects (i.e. the parasitic fly *Wohlfahrtia magnifica* (Reed et al., 2024)) and could have been derived from the supplementation with BSFL.

The observed increase of several bacterial genera following BSFL supplementation compared to the control suggests that BSFL significantly influences the microbiota in the excreta. Similar shifts in specific bacterial members have been documented in poultry with diets supplemented with live BSFL and *T. molitor,* where increased in *Clostridium*, Saccharibacteria and Victivallaceae were noted, with *Collinsella* being predominant in *T. molitor* supplementation (Colombino et al., 2021). In our study, the consistent increase of specific genera across all time points and supplementation levels suggests a direct association with BSFL supplementation. For example, *Klebsiella, Actinomyces, Proteus, Enterococcus* and *Morganella* are frequently found in BSFL being part of the core microbiota and are associated with various metabolic functions, including protein degradation and fermentation (Vandeweyer et al., 2023; Silvaraju et al., 2024). In particular, *Actinomyces* and *Klebsiella*, both commonly present in the gut of BSFL grown on various substrates (Lin and Shelomi, 2024), showed consistent increase, likely due to the repeated introduction trough the BSFL supplementation. *Actinomyces* are frequent inhabitants of the gut, for example they are part of the normal human oral and gastrointestinal microbiota, where they play a role in breaking down complex organic materials, aiding in nutrient absorption, and help maintain a balanced microbial community (BiologyInsights, 2024). *Actinomyces* are also quite common in soil ecosystems, where they decompose organic matter, contributing to nutrient cycling and soil fertility, where they may produce antibiotics that inhibit the growth of competing microorganisms (BiologyInsights, 2024).

Klebsiella is frequently found in BSFL, is known for nitrogen-fixing properties and can become opportunistic pathogens under immune-compromised conditions (Silvaraju et al., 2024).

Other genera such as *Miniimonas, Morganella, Proteus, Leucobacter,* and *Citrobacter* previously identified in BSFL gut (Zhineng et al., 2021; Reed et al., 2024; Lin and Shelomi, 2024), also showed increased abundance at some specific sampling points and may be directly associated to their continuous intake with BFSL supplementation.

The inconsistent detection of some bacterial genera associated with BSFL supplementation across different sampling periods likely reflects the dynamic and responsive nature of the gut microbiota (Allaband et al., 2019). Some variations may also be associated to variations in the BSFL (Silvaraju et al., 2024) influenced by several biological factors, including the age and developmental stage of the host, changes in diet composition or intake over time, and the transient colonization of certain microbes that do not establish long-term residence in the gut.

Miniimonas is knowns for its role in cellulose degradation within insect gut (Xiang et al., 2024), while *Proteus* spp. and *Morganella* spp. are widespread in the environment and are part of the normal microbiota of human gastrointestinal tract, where can also act as unusual opportunistic pathogens (O'Hara et al., 2000; Li et al., 2016). *Leucobacter* spp. is widespread across plants, animals, and free-living environments reflecting a remarkable niche versatility and may act as a symbiont in nematodes (Bates et al., 2021). *Citrobacter* spp., increased may be linked to the high lipid content of BFSL, as similar trends were observed in fish, where it enhanced energy harvest by promoting lipid accumulation and increasing triglyceride absorption and intestinal permeability (Zhang et al., 2020). Furthermore, the increases at the last sampling point in *Pseudogracilibacillus, Cerasibacillus*, and *Hespellia*, all detected in BSFL or insect-based diet or insect-derived products (Raimondi et al., 2020; Milanović et al., 2020; Pei et al., 2022), further support the association between these genera and BSFL supplementation (Raimondi et al., 2020; Kim et al., 2024). On the other hand, some other genera may be associated to indirect effect of the nutritional content of BSFL and/or an improved quality of the diet. *Oblitimonas*, previously identified in BSFL and associated to the inhibition of pathogens (Zhang et al., 2022), highlights a potential antimicrobial benefit of BSFL supplementation. The rise in *Georgenia* abundance aligns with findings from white-feathered broilers dietary supplementation with *Eucommia ulmoides* bark extract, which improved caecal microbiota composition (Lu et al., 2024) and thus considered a positive effect of BSFL supplementation. *Anaerovorax* may be linked to the higher proteins provided by BSFL, as it has been associated with protein-rich diets in cattle (Lau et al., 2018). The increase of *Urmitella* and *Sedimentibacter* is more difficult to be interpreted, because of limited research on these genera (Lechner, 2015; Pham et al., 2017). A positive outcome of BSFL supplementation was the decrease of *Brachybacterium*, a genus found significantly more abundant in faeces, litter, feeder, and waterer swabs from commercial farms and also linked to low-performing broiler flocks (Muyyarikkandy et al., 2023). *Lactonifactor* could be influenced by diet composition rather than direct gut contamination from BSFL in fact it was found to increase with *Amaranthus hypochondriacus* stem and leaf powder supplementation in broilers (Sun et al., 2024) and increased in the chicken manure after feeding a protein-rich diet (Xu et al., 2018). Interestingly the BSFL supplementation in our study displayed an high concentration of *Lachnoclostridium* in the excreta at the last sampling point and may indicate a positive effect since it increase was previously associated with higher body weight in chickens due to its positive role in energy efficiency and muscle fiber diameter (Lei et al., 2022). Contrary to previous studies that reported a decrease in laying hens (Borrelli et al., 2017), we observed an increase in *Fusobacterium* with BSFL supplementation. Similarly, *Intestinimonas* decreased in our study, contrasting with previous findings where ad libitum access of laying hens to BSFL larvae significantly increased its abundance (Huang et al., 2024). The increase in *Salmonella* at the last sampling point, which is in contrast with previous findings (Erickson et al., 2004; Lee et al., 2018), requires further studies to clarify potential risks associated to BSFL supplementation.

Regarding the dynamics over time, only *Actinomyces* and *Klebsiella* consistently increased, while Miniimonas, Georgenia, Urmitella, and Fusobacterium showed varied increases during time, indicating that BSFL supplementation might create a favorable environment for these microbes at specific growth stages (Kim et al., 2021). The *Leucobacter, Sedimentibacter, Proteus, Intestinimonas*, and *Anaerovorax* increase at week 20 suggests an initial boost in these genera, possibly due to the initial adaptation of the gut microbiota to the new diet, followed by a stabilization. The increase in *Oblitimonas* and decrease in *Brachybacterium* at week 28 could indicate a shift in microbial dynamics as the chickens mature, while the increase at week 34 of *Cerasibacillus, Hespellia, Lactonifactor, Virgibacillus,* and *Morganella* suggests these genera benefit from prolonged BSFL supplementation. The increase with 15 % BSFL at week 20 and both levels at week 34 indicates that *Lachnoclostridium* may have a role only at lower level of supplementation and/or with longer exposure.

Considering the enrichment effect offered by BSFL larvae, the supplemented groups of our study can be assimilated to low feather pecking animals (LFP) since the peaking behaviour was stimulated by insects and there was no tendency to feather pecking. In accordance with our data, in LFP animals was found a reduced presence of *Clostridiae* and an increased number of *Lactobaccillacae*, comparing these treatments with high feather pecking (False Discovery Rate < 0.05, Mann–Whitney comparisons) (Birkl et al., 2018). Similarly, Benzertiha et al. (2019) evaluated the effects of insect full-fat meal inclusion (0.2 % or 0.3 % *T. molitor*) in broiler chicken diet on the microbiota community. In accordance with our date, the insect inclusion significantly decreased the caecal counts of the *Bacteroides–Prevotella* cluster, compared with the control, concluding the positive effect of insect meals on reducing potentially pathogenic bacteria for broilers, such as *Bacteroides–Prevotella* cluster and *Clostridium perfringens*.

Conclusions

The innovative aspect of this research lies in the use of live BSFL as a dietary supplement for laying hens. The analysis of the hens' performance and integument scores showed no negative effects of this novel ingredient in either of the two experimental groups. Most parameters were influenced by time, as the hens were observed between 16 and 34 weeks of age, during which they underwent a natural growth phase. The corticosterone concentration in the excreta indicated lower stress levels in the BSFL-supplemented groups, suggesting that live BSFL supplementation might be associated with reduced stress indicators. Furthermore, the supplementation did not significantly impact the dominant genera of the microbiota, highlighting a stable core microbiota. However, the bacterial community did show some structural differences, possibly influenced by age and/or environmental factors. BSFL supplementation can significantly modulate gut microbiota composition, with possible implications for poultry health, nutrient absorption and energy efficiency.

Further research could explore the rearing of larvae on different agri-food substrates to determine whether insect nutrition has a corresponding impact on hen performance.

In summary, feeding live BSFL to laying hens may represent a sustainable and effective feed source that does not negatively impact performance while enhancing animal welfare. However, since microorganisms from BSFL can colonize the animals' gut, the potential for pathogen transmission should be acknowledged. This highlights the importance of monitoring the sanitary quality of the larvae and maintaining strict hygiene standards for the rearing substrates. Overall, this approach represents a significant step toward a more resilient and eco-friendly food production system in poultry nutrition.

Availability of data and materials

The data obtained and analysed during the current study are available from the corresponding author on reasonable request.

Disclosures

The authors declare no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.105418.

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