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RESEARCH ARTICLE

Nutritional and bacterial microbiota implications of partial inclusion of live *Hermetia illucens* larvae in "Collo Nudo Italiana" chicken diets

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Abstract

The increasing demand for sustainable protein sources in poultry production has intensified interest in insects as alternative feed ingredients. This study evaluated the effects of partially replacing conventional dietary protein sources with live *Hermetia illucens* larvae in chicken diets, focusing on nutrient intake, growth performance, health status, gut microbiota, and carcass fat fatty acid (FA) composition. Three independent feeding trials were conducted using female "Collo Nudo Italiana" chickens reared under extensive conditions. Chickens were assigned to either a control or an experimental diet administered *ad libitum*, and feed intake was monitored. In the experimental diet, live *H. illucens* larvae replaced approximately 20% of dietary protein, significantly increasing dry matter and protein intake, indicating high palatability of the larvae. However, body weight (BW) and growth performance were not significantly affected, and no adverse effects on health status or welfare indicators were observed. Caecal bacterial microbiota analysis revealed no differences in alpha diversity between groups ($p > 0.05$), whereas beta diversity showed a significant ($p < 0.05$) but moderate shift in microbial community structure. The genera *Staphylococcus*, *Brevibacterium*, and *Alloprevotella* were enriched in treated animals, whereas a relative abundance of *Pseudoflavonifractor*, *Tyzzerella* and *Shuttleworthia* was observed in the control group. Inclusion of live larvae influenced carcass fat composition, increasing medium-chain saturated FAs characteristic of *H. illucens* lipids and reducing polyunsaturated FAs, particularly n-3 fatty acids. These findings support the potential of live insect larvae as a sustainable feed component in circular poultry production systems, highlighting the need for optimized dietary formulations to balance nutritional outcomes.

Keywords

alternative protein sources – circular economy – gut microbiota – sustainable poultry production

1 Introduction

Circularity in resource use is necessary to sustain the world's growing population in the face of shrinking arable land and current climate change (Hoffmans *et al.*, 2024). The FAO states that the trend until 2050 is characterized by an increase in meat consumption (OECD/FAO, 2022). This has led to an intensification of livestock production and, consequently, an expected increase in feed supply of 1.3 billion tonnes of dry matter (DM) (Parisi *et al.*, 2020). In poultry farming, feed production significantly contributes to the overall environmental impact, largely because of the inclusion of protein-rich components, such as soybean meal (Grigore *et al.*, 2025; Lanza *et al.*, 2025; Pope *et al.*, 2023). Therefore, the pursuit of alternative protein sources that can decrease the dependence on standard crops while ensuring the maintenance of animal performance is a crucial challenge for achieving sustainable poultry production (Allegretti *et al.*, 2018).

Insects have been increasingly proposed as alternative feed ingredients because of their high nutritional value and ability to efficiently convert low-value organic substrates into high-quality biomass (Kazemi and Valizadeh, 2025; Maroušek *et al.*, 2023; Pinotti *et al.*, 2019). Among the various species investigated, *Hermetia illucens* (Diptera Stratiomyidae) (black soldier fly) larvae are regarded as the principal arthropod species for global animal feed production because of their amino acid profile, lipid content, and suitability for large-scale production in a circular economy (Reguzzi *et al.*, 2021; Madau *et al.*, 2020). Different studies have demonstrated that including *H. illucens* larvae meal in poultry diets does not negatively impact growth performance or health (Iqbal *et al.*, 2025; Lee *et al.*, 2022; Maurer *et al.*, 2016). However, most studies have focused on the inclusion of processed insect meals, whereas information on the use of live larvae in chicken diets is limited (Fiorilla *et al.*, 2024; Schiavone and Castillo, 2024; Lu *et al.*, 2022; Veldkamp and Bosch, 2015).

The inclusion of insect-derived ingredients in diets can influence meat and fat quality traits, particularly fatty acid (FA) composition (Gasco *et al.*, 2019). The lipid fraction of *H. illucens* larvae is characterized by a high proportion of medium-chain saturated FA (Cattaneo *et al.*, 2023). These FA can be assimilated into animal tissues, thereby altering the nutritional profile of poultry meat (Schiavone *et al.*, 2018). In addition, dietary changes can affect the gut microbiota, which in turn affects nutrient utilization, immune function, and overall animal health (Biasato *et al.*, 2019; Yadav and Jha, 2019). Therefore, a holistic assessment of growth performance, meat quality, and gut microbiota is essential to thoroughly understand the implications of feeding strategies involving *H. illucens* larvae.

The present study aimed to evaluate the effects of partially replacing conventional dietary protein sources with live *H. illucens* larvae in the chicken diet. The effects of larvae supplementation on nutrient intake, growth performance, carcass characteristics, FA composition of carcass fat, and cecal microbiota were investigated across three independent experimental trials.

2 Materials and methods

The project

The study was structured into three independent experimental trials carried out between July and October 2021 at a commercial poultry farm located in the province of Piacenza (Italy), specialising in the rearing and commercialization of rustic chickens of the "Collo Nudo Italiana" breed.

Experimental design

Female chickens of the "Collo Nudo Italiana" (Aglietto Natura, Bianzè, Italy) breed, a local genotype comparable to the international "Naked Neck" breed, were employed in this study. This breed was selected because of its recognized hardiness, adaptability to extensive rearing systems, and suitability for alternative dietary formulations. Three independent trials were conducted, each lasting approximately 30 days in July, September, and October. At the beginning of each trial, chickens were 48 days (average BW 985 g; trial 1), 48 days (average BW 1334 g; trial 2), and 83 days (average BW 1,630 g; trial 3). In each trial, 24 chickens were allocated to two experimental groups: 12 to the "control" (CTR) and 12 to "treatment" (TRT), with each group further subdivided into four pens containing three chickens. The CTR group was fed a conventional diet consisting of ground maize (63.64% DM) and a commercial complementary feed (NP32SS-Nucleo Polli 2P Oscar, 36.36% DM) for chickens (Table A1 in the Appendix). The TRT group received the same basal diet as the CTR group, namely ground maize (64.46% DM) and complementary feed (26.06% DM), supplemented with live *H. illucens* larvae, provided daily, and replacing approximately 20% of the protein fraction (9.48% DM). The two diets were formulated to be isoproteic, with a crude protein content of 18% and isocaloric, providing approximately 2800 kcal of metabolizable energy (ME) per kg of diet. Diet formulation

was based on crude protein content to reflect practical feeding conditions; therefore, no correction based on digestible protein coefficients was applied. Both diets were administered *ad libitum*, and feed intake was monitored.

Larval production of Hermetia illucens

The larvae used in the experimental trials were reared in a dedicated room on the campus of the Università Cattolica del Sacro Cuore (Piacenza, Italy). The growth substrate consisted of fresh plant by-products, mainly unsold fruits and vegetables and selected agro-industrial residues, thereby valorising these waste streams within the circular economy framework. The materials were chopped and homogenized to ensure substrate uniformity of the mixture. The substrate was periodically renewed to maintain optimal hygiene and promote larval growth. Rearing was carried out in climate-controlled chambers at 27-30 °C, 65-70% relative humidity, and under a standard photoperiod (16:8 h light:dark cycle). The mature larvae were collected either manually or by sieving at 14-15 days of development, when they had reached an average BW of 80-100 mg and the highest possible crude protein content before pupation (Liu *et al*., 2017). After collection, the larvae were washed, superficially dried and then delivered immediately to the chicken farm for distribution. The required quantities were weighed and delivered to the farm each day, where they were manually mixed with the feed for the TRT group immediately before its administration.

Housing and chicken management

Chickens were housed in pens of three animals each, constructed with wire mesh and a top cover, and were equipped with permanent cereal husk bedding. Each pen was provided with automatic drinkers, allowing continuous water availability, and with raised feeders with inward-curved edges to prevent larval escape and reduce the chickens' scavenging behaviour. The indoor temperature and relative humidity within the housing facility were continuously monitored using a data logger. Body weight was recorded individually at the beginning of each trial, at 5-day intervals, and at the end of the experimental period. During Trial 3, however, BW measurements could not be systematically recorded because stricter COVID-19 pandemic restrictions limited access to the poultry house to a single operator at a time, whereas the experimental protocol required two operators to safely handle and weigh the birds. Although some partial measurements were collected, the dataset was incomplete and therefore unsuitable for reliable statistical analysis. Feed intake was measured daily on a pen basis as the difference between the amount of feed offered and the residual feed remaining in the feeders. The protein and DM intakes were calculated based on the known chemical composition of the commercial feed and *H. illucens* larvae administered to the chickens. In addition, clinical and behavioural observations were performed daily, including assessment of plumage condition, presence of lesions, comb and wattle status, aggressive behaviour, general vitality, and faecal consistency.

DNA extraction, 16S ribosomal DNA amplification and sequencing

In each of the three experimental trials, the intestines were collected at slaughter from six CTR and six TRT chickens for analysis. Intestinal contents from the three gut sections were sampled to investigate the gut microbiota composition. All samples were immediately stored at –80 °C until further analysis and were subsequently analysed using Next-Generation Sequencing (NGS) techniques. The FastDNA™ SPIN Kit for Soil (MP Biomedicals, Switzerland) was used to extract total bacterial DNA from 50 mg (wet weight) of intestinal content, according to the manufacturer's instructions. DNA concentration was quantified using the Qubit HS dsDNA assay kit (Life Technologies, Carlsbad, CA, USA), and its quality was checked by agarose gel electrophoresis. Amplification was conducted as previously described (Patrone *et al*., 2018). Briefly, DNA amplification was carried out using the primers 343F and 802R, targeting the V3-V4 hypervariable regions of the bacterial 16S rRNA gene. A unique seven-nucleotide barcode was added to the forward primer to allow sequence assignment to individual samples during downstream bioinformatic analysis. After quantification, the amplicons were mixed to obtain a pool in which the PCR products from each sample were present at an equimolar concentration. The DNA from this pool was then purified using the DNA Clean and Concentrator™-5 kit (Zymo Research, Irvine, CA, USA). Sequencing was performed at Fasteris (Geneva, Switzerland) using the Illumina MiSeq approach in the 2 × 300 bp paired-end mode. Trimmomatic version 0.32 (http://www.usadellab.org/cms/index.php?page=trimmomatic) (Bolger *et al*., 2014) was used to filter raw reads and exclude low-quality sequences (quality score ≥ 30). The 16S rRNA reads were taxonomically classified based on the Silva SSU reference database.

TABLE 1 Dry matter intake, protein supply, and body weight of chickens in the three experimental trials

	Treatment		Effect			SE
	CTR	TRT	Treatment (Trt)	Date	Trt × Date	
Trial 1						
Total protein content (g DM)	59.52	70.82	0.001	<0.001	<0.001	0.010
Dry matter intake (g)	240.64	272.52	0.005	<0.001	<0.001	0.009
Body weight (kg)	1.29	1.26	0.569	<0.001	0.310	0.030
Trial 2						
Total protein content (g DM)	68.92	84.34	<0.001	<0.001	<0.001	0.010
Dry matter intake (g)	278.66	324.55	0.001	<0.001	<0.001	0.008
Body weight (kg)	1.64	1.66	0.730	<0.001	0.031	0.048
Trial 3						
Total protein content (g DM)	82.21	119.64	<0.001	<0.001	<0.001	0.010
Dry matter intake (g)	332.39	460.39	<0.001	<0.001	<0.001	0.007
Body weight (kg)	–	–	–	–	–	–

Total protein content and dry matter intake data were $\log_{10}$-transformed before statistical analysis.

Fatty acids profile

Fatty acid methyl esters (FAME) were prepared from approximately 0.05 g of carcass fat sample collected at slaughter from six CTR and six TRT chickens using the direct transesterification procedure described by O'Fallon *et al.* (2007). The resulting FAME were separated and quantified by gas chromatography using a Shimadzu GC system (model 2025, Shimadzu, Kyoto, Japan) equipped with an automatic sampler (AOC-20s), flame ionization detector (FID), and CP-Select CB capillary column specifically designed for FAME analysis (100 m × 0.25 mm internal diameter, 0.25 μm film thickness; Chrompack, Varian, Palo Alto, CA, USA). A volume of 1 μl was injected in split mode, with hydrogen as the carrier gas at a constant flow rate of 1.5 ml/min. Both the injector and detector temperatures were maintained at 250 °C. The oven temperature program started at 60 °C for 2 min, increased to 170 °C at a rate of 10 °C/min with a holding time of 35 min, and then ramped to 240 °C at 4 °C/min for an additional 9.5 min. Chromatographic data acquisition and processing were performed using the LabSolutions Lite software (version 5.82, Shimadzu, Milan, Italy). Individual FAs were identified by comparing retention times with those of certified external standards (Supelco 37 Component FAME Mix, PUFA-3 Menhaden oil, and conjugated octadecadienoic acid; Sigma, St. Louis, MO, USA). The results were expressed as percentages of total FAs and calculated from peak areas corrected using the appropriate response factors in accordance with the AOAC method 963.22 (AOAC, 2000).

Statistical analysis

Different statistical approaches were applied depending on the data structures. Statistical analyses were performed separately for each trial. Data on dry matter intake (DMI), total protein content (on a DM basis), and BW were $\log_{10}$-transformed before analysis to improve the normality and stabilize the variance. For each variable, a linear mixed model with repeated measures was fitted, including treatment (CTR vs TRT), sampling date, and their interactions as fixed effects. Pen was considered the experimental unit and included as a random effect, with sampling date treated as the repeated factor within pen. The *p*-values for the fixed effects are reported in Table 1. Statistical significance was set at $p < 0.05$. All analyses were performed using JMP® software (version 17.0.0; SAS Institute, Cary, NC, USA).

MicrobiomeAnalyst 2.0 (Dhariwal *et al.*, 2017; R core team, 2020) was used for statistical analysis of the sequences. This platform was also used to estimate alpha diversity based on the observed species, Chao 1, and Shannon indices. The results are displayed across individual samples for each animal group and are additionally summarized using box plots for each group. Differences among samples were assessed using the Bray-Curtis dissimilarity metric and pairwise dissimilarities were visualized through principal coordinates analysis (PCoA) (Bray and Curtis, 1957). Permutational Multivariate Analysis of Variance (PERMANOVA) was performed to investigate whether the community composition differed significantly between the two groups of animals. The edgeR algorithm with a *p*-value cut-off

TABLE 2 Fatty acid composition (% of total fatty acids) of chicken carcass fat in the three experimental trials

FA (% of total FA)	Larvae	Trial 1				Trial 2				Trial 3			
		CTR	TRT	SEM	*p*-value	CTR	TRT	SEM	*p*-value	CTR	TRT	SEM	*p*-value
Protein (%)	31.81	–	–			–	–			–	–		
Fat (%)	18.97	–	–			–	–			–	–		
C 10:0	0.83	0.01	0.03	0.004	0.007	0.01	0.04	0.009	0.065	0.01	0.04	0.006	0.001
C 12:0	46.01	0.03	1.80	0.340	0.010	0.37	3.92	0.968	0.086	0.12	3.47	0.547	0.001
C 14:0	7.99	0.75	1.30	0.113	0.016	0.80	1.81	0.280	0.091	0.61	1.42	0.130	0.000
C 14:1	0.23	0.12	0.24	0.021	0.000	0.12	0.24	0.022	0.002	0.10	0.20	0.018	0.001
C 15:0	0.23	0.11	0.10	0.004	0.641	0.09	0.09	0.006	0.710	0.08	0.07	0.003	0.045
C 16:0	14.23	25.75	26.55	0.382	0.319	25.66	25.94	0.556	0.822	25.17	25.32	0.231	0.765
C 16:1	2.87	3.76	5.59	0.382	0.010	3.85	5.48	0.485	0.104	3.55	4.82	0.361	0.082
C 17:0	0.22	0.18	0.15	0.009	0.036	0.15	0.14	0.009	0.345	0.15	0.11	0.008	0.004
C 18:0	2.88	8.04	5.89	0.478	0.027	7.05	5.88	0.299	0.045	7.38	6.14	0.403	0.130
C 18:1n9c	12.79	39.12	38.99	0.367	0.873	38.86	36.88	0.841	0.263	42.79	41.07	0.435	0.041
C 18:1cis11	0.60	1.70	2.00	0.068	0.019	1.69	1.79	0.061	0.464	1.72	1.70	0.041	0.781
C 18:2n6c	8.68	18.85	15.76	0.665	0.012	19.63	16.28	0.954	0.094	16.85	14.36	0.660	0.056
C 18:3n6	0.00	0.10	0.11	0.005	0.223	0.12	0.09	0.008	0.011	0.09	0.09	0.007	0.735
C 18:3n3	1.08	0.61	0.62	0.018	0.884	0.67	0.60	0.036	0.403	0.59	0.48	0.035	0.140
C 20:0	0.10	0.09	0.06	0.009	0.081	0.08	0.06	0.005	0.099	0.08	0.05	0.006	0.052
CLA tot.	1.22	0.04	0.09	0.011	0.019	0.04	0.08	0.010	0.078	0.04	0.14	0.016	0.000
C 20:1	0.00	0.33	0.29	0.013	0.088	0.31	0.26	0.011	0.021	0.30	0.24	0.013	0.010
C 20:2	0.00	0.10	0.08	0.006	0.112	0.11	0.09	0.008	0.246	0.07	0.05	0.005	0.058
C 20:3n6	0.00	0.08	0.10	0.006	0.060	0.09	0.09	0.005	0.684	0.06	0.06	0.004	0.980
C 20:3n3	0.00	0.01	0.01	0.001	0.052	0.01	0.01	0.001	0.199	0.01	0.00	0.001	0.048
C 20:4n6	0.00	0.15	0.18	0.015	0.344	0.20	0.17	0.018	0.579	0.16	0.12	0.021	0.323
C 22:0	0.06	0.01	0.01	0.001	0.140	0.01	0.01	0.001	0.140	0.01	0.01	0.001	0.066
C 22:1n9	0.00	0.01	0.01	0.001	0.172	0.01	0.01	0.000	0.004	0.01	0.01	0.001	0.061
C 20:5n3	0.00	0.00	0.01	0.001	0.149	0.01	0.01	0.001	0.480	0.01	0.00	0.002	0.334
C 24:0	0.00	0.05	0.04	0.003	0.438	0.05	0.04	0.005	0.693	0.04	0.03	0.004	0.125
C 22:6n3	0.00	0.01	0.01	0.001	0.508	0.01	0.01	0.001	0.725	0.01	0.00	0.003	0.493
SFA	72.53	35.02	35.93	0.629	0.501	34.27	37.93	1.033	0.090	33.64	36.65	0.610	0.006
MUFA	16.49	45.03	47.11	0.660	0.131	44.85	44.66	1.162	0.939	48.47	48.03	0.571	0.724
PUFA	10.99	19.95	16.97	0.667	0.017	20.88	17.41	1.006	0.102	17.90	15.32	0.695	0.061
UFA	27.47	64.98	64.07	0.629	0.501	65.73	62.07	1.033	0.090	66.36	63.35	0.610	0.006

adjusted to 0.05 was used to identify significant differences in the abundance of different taxa between the two groups of samples. The relative abundance of all taxa in the two groups of animals was analyzed using linear discriminant analysis (LDA) and effect size analysis (LEfSe) using MicrobiomeAnalyst 2.0.

The FA composition of chicken carcass fat was analysed separately for each experimental trial (Table 2). For each FA and the main FA classes, saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA), and total unsaturated FA (UFA), the mean values were calculated at the pen level, and dietary groups (CTR and TRT) were compared using an independent samples *t*-test with Welch's correction. The results were expressed as percentages of total FA, and variability was reported as the standard error of the mean (SEM). Statistical significance was set at $p < 0.05$.

3 Results

Body weight, feed intake, protein and dry matter intake

The results below describe the impact of the treatments on DM and protein content, as well as growth performance (Table 1). Across the three trials, significant treatment × date interactions were observed for both total protein content and DMI ($p < 0.001$), indicating that the effect of larvae inclusion on nutrient intake varied

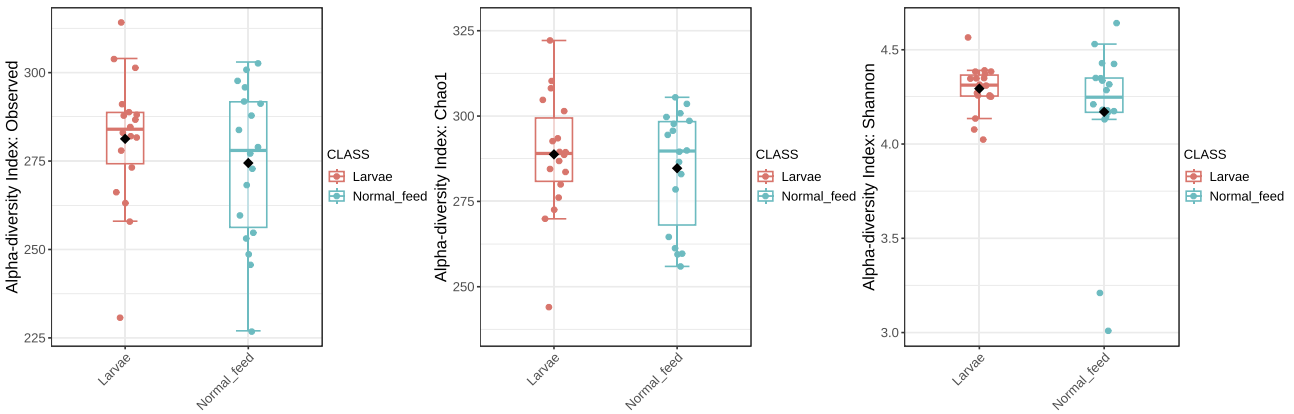

FIGURE 1 Alpha diversity analysis of caecal samples from the two animal groups.

across the sampling times (Table 1). In all trials, the TRT group showed higher values than the CTR group. Specifically, the total protein content on a DM basis increased from 59.52 g in CTR to 70.82 g in TRT in Trial 1 (p = 0.001), from 68.92 to 84.34 g in Trial 2 (p < 0.001), and from 82.21 g to 119.64 g in Trial 3 (p < 0.001). In addition, DMI was higher in the TRT group, rising from 240.64 g to 272.52 g in Trial 1 (p = 0.005), from 278.66 to 324.55 g in Trial 2 (p = 0.001), and from 332.39 to 460.39 g in Trial 3 (p < 0.001). In all cases, both the treatment and date effects were significant (p < 0.001), confirming the impact of dietary treatment and sampling time on nutrient intake. Concerning body weight, a significant treatment × date interaction was detected only in Trial 2 (p = 0.031), suggesting that the growth response to dietary treatment varied over time in this trial. However, no significant overall treatment effects were observed in either Trial 1 or Trial 2 (p = 0.569 and p = 0.730, respectively). In both trials, the date effect was significant (p < 0.001). Body weight data were not available for Trial 3 (see Materials and Methods for details).

Clinical and behavioural observations

Throughout the experimental period, no differences were observed between the CTR and TRT groups. The overall health status of the animals was good in both groups, with no evidence of disease, mortality, or clinical signs attributable to the dietary treatment. The feathers were normal, the comb and wattles showed no pathological alterations, and the droppings were consistently well-formed without diarrheal episodes. The inclusion of *H. illucens* larvae in the diet did not negatively affect the welfare or clinical condition of the chickens.

Bacterial microbiota

Alpha and beta diversity analyses were conducted to evaluate the differences in the diversity of microbial communities in the stool samples collected from the two groups of animals. Alpha diversity based on the observed species, Chao 1 and Shannon indices revealed no significant differences between the two experimental groups. The p-values obtained were 0.32, 0.49 and 0.24 for the observed species, Chao 1, and Shannon indexes, respectively (Figure 1). Results of alpha diversity analysis of microbiota as evaluated by observed species, Chao 1 and Shannon Diversity Index. The caecal microbiota diversity in animals receiving the TRT diet was not significantly different from that of CTR animals.

Regarding beta diversity, PERMANOVA showed significant differences between the two groups of animals (R^2 = 0.055; FDR = 0.007), even if there was no clear visual separation in beta diversity of caecal microbiota between the two groups of animals (Figure 2).

The edgeR algorithm revealed that only the *Brevibacteriaceae* family was significantly more abundant (FDR = 0.008) in the TRT group than in the CTR group. At the level of genera, *Staphylococcus* (FDR = 2.1159E-6), *Brevibacterium* (FDR = 0.003) and *Alloprevotella* (FDR = 0.025) were significantly more represented in the cecal microbiota of TRT animals than in CTR animals. The latter, on the other hand, showed a higher presence of *Pseudoflavonifractor* (FDR = 0.003), *Tyzzerella* (FDR = 0.02) and *Shuttleworthia* (FDR = 0.033).

Moreover, LEfSe analysis confirmed that taxonomic enrichment of *Staphylococcus* characterized TRT animals, whereas *Pseudoflavonifractor* and *Tyzzerella* were associated with CTR animals (Figure 3).

Considering the potential concern associated with an increase in *Staphylococcus*, we conducted a detailed examination of the relative abundance data for this genus. Specifically, the mean relative abundances ob-

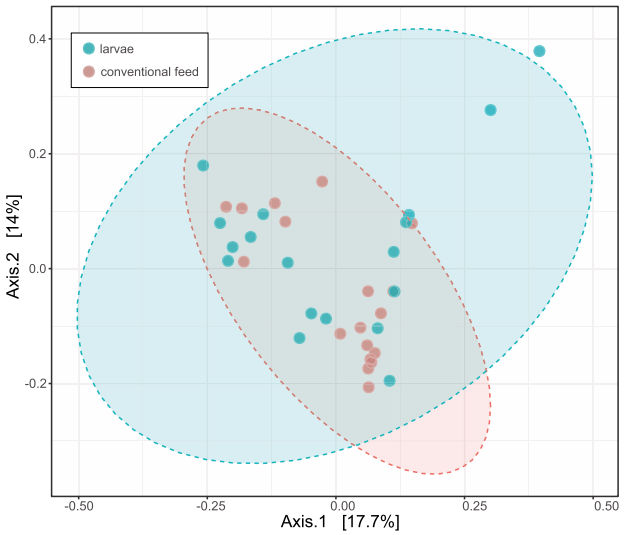

FIGURE 2 Principal coordinate analysis of Bray-Curtis dissimilarity between the caecal microbiota of animals receiving larvae-containing feed and animals receiving conventional feed. Each point represents a different individual in the study.

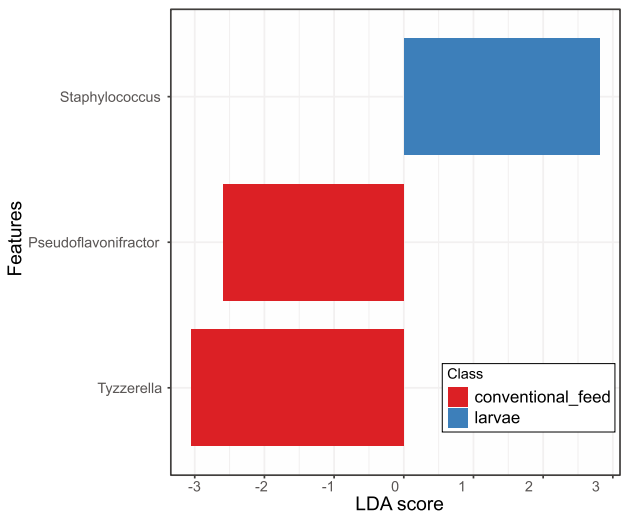

FIGURE 3 Linear discriminant analysis (LDA) effect size (LEfSe) analysis showing microbial taxa that characterize animals fed with larvae-enriched feed and those fed with conventional feed based on LDA score > 2 and $p < 0.05$.

served in the control group (0.00018%) and in the treated group (0.00057%) indicate that the *Staphylococcus* population is maintained at very low levels in both groups, consistent with values reported in the literature (Ayala *et al.*, 2023).

Fatty acids

The FA composition of chicken carcass fat differed between the CTR and TRT groups across the three experimental trials, with consistent effects observed for several individual FA and the main FA classes (Table 2).

In all trials, the inclusion of live *H. illucens* larvae in the diet increased the specific SFA, particularly the medium-chain FA characteristics of larval lipid profiles. In Trial 1, the concentrations of C10:0, C12:0, and C14:0 were higher in the TRT group than in the CTR group ($p = 0.007$, 0.010 and 0.016, respectively). A similar pattern was observed in Trials 2 and 3, where C12:0 and C14:0 increased in the TRT group, with values approaching significance in Trial 2 ($p = 0.086$ and 0.091, respectively) and differences in Trial 3 ($p \leq 0.001$). In Trial 2, the C10:0 content tended to be higher in the TRT group ($p = 0.065$). Consequently, the total SFA content was higher in the TRT group in Trial 3 ($p = 0.006$) and tended to increase in Trial 2 ($p = 0.090$). Regarding MUFA, most individual MUFA levels did not differ between the treatments ($p > 0.10$). However, some differences were detected in specific FAs. In Trial 1, C14:1 and C16:1 were higher in the TRT group ($p < 0.001$ and $p = 0.010$, respectively), whereas C18:1n9c, the predominant MUFA, was not affected by dietary treatment in Trials 1 and 2 and showed a reduction in the TRT group in Trial 3 ($p = 0.041$). On the whole, the total MUFA content was not influenced by the dietary treatment in any of the trials. In contrast, the inclusion of larvae reduced PUFA content. In Trial 1, the TRT group exhibited a lower proportion of total PUFA than the CTR group ($p = 0.017$), mainly due to the reduction in C18:2n6c ($p = 0.012$). In Trials 2 and 3, C18:2n6c tended to decrease in the TRT group ($p = 0.094$ and $p = 0.056$, respectively), supporting the observed reduction in total PUFA. Consistent with the changes observed in SFA and PUFA, the total UFA fraction was reduced in the TRT group in Trial 3 ($p = 0.006$) and tended to be lower in Trial 2 ($p = 0.090$).

4 Discussion and conclusion

The present study evaluated the effects of partially replacing conventional dietary protein sources with live *H. illucens* larvae in chicken diets by assessing productive performance, health status, gut microbiota, and carcass FA profiles across three independent experimental trials conducted under different seasonal and environmental conditions.

The observed increase in nutrient intake in live larvae-fed chickens suggests that live larvae are a highly palatable dietary component, as noted by Lu *et al.* (2022). This effect is likely attributable to the physical characteristics and sensory properties of larvae, as mentioned by Sverguzova *et al.* (2021), which may

stimulate natural foraging behaviour in chickens (Veldkamp and Bosch, 2015) and enhance voluntary feed consumption (Schiavone and Castillo, 2024). Similar behavioural responses have been described in poultry fed structurally diverse feed components (Shiavon *et al.*, 2018), supporting the hypothesis that feed form, in addition to nutrient composition, plays a role in regulating intake (Richards and Proszkowiec-Weglarz, 2007). However, our results explain the lack of a corresponding improvement in growth performance, showing that increased nutrient intake does not necessarily translate into enhanced BW gain (Lanza *et al.*, 2025), particularly in slow-growing or rustic genotypes (N'dri *et al.*, 2007). These findings are in line with previous studies (Schiavone and Castillo, 2024; Lu *et al.*, 2022; N'dri *et al.*, 2007) that reported unchanged growth performance in broilers fed insect-based diets and underline the complexity of growth regulation, which is influenced by genetic, environmental, and metabolic factors (Lee *et al.*, 2022). In addition, a significant treatment × date interaction observed for BW suggests that growth responses may vary across the experimental period, despite the absence of an overall treatment effect on BW. In this respect, previous studies on slow-growing indigenous breeds, such as Bianca di Saluzzo chickens, have reported improved growth performance following *H. illucens* larvae supplementation, highlighting a breed- and context-dependent productive response to insect-based feeding strategies (Fiorilla *et al.*, 2024). It should also be noted that BW data were not available for Trial 3, which may limit the interpretation of the performance consistency across all trials. It should also be considered that the three trials were conducted at different physiological stages, particularly Trial 3, in which birds were older (83 days) and heavier at the start of the experimental period. Age-related differences in metabolic activity, feed intake capacity, and lipid deposition patterns may partly explain variations in absolute intake values and FA responses observed among trials. However, because each trial was analysed independently, these baseline differences do not affect the internal comparison between dietary treatments. Another limitation of the present study concerns experimental replication. Because the pen was considered the experimental unit, each treatment included four pens per trial (n = 4). A post-hoc sensitivity analysis performed with G*Power version 3.1.9.4 (F test, one-way ANOVA, $\alpha = 0.05$, power = 0.80, total sample size = 8, two groups) indicated that this design was powered to detect only large effects (minimum detectable effect size: $F = 1.19$; equivalent to Cohen's $d \approx 2.38$). Therefore, the absence of statistically significant differences in BW should be interpreted as the absence of detectable effects under the present experimental conditions rather than definitive evidence of equivalence between diets, and larger studies would be required to confidently exclude small differences in growth performance.

In this study, the absence of negative effects on health and animal welfare parameters confirmed the safety of live *H. illucens* larvae in chicken diets, as mentioned by Lee *et al.* (2022) and Bellezza Oddon *et al.* (2021). The results suggest that live larvae do not appear to adversely affect gut function, immune status, or general animal well-being, as also in previous studies on insect meals, which are a feeding strategy that is closer to natural dietary behaviours (Schiavone and Castillo, 2024; Bongiorno *et al.*, 2022). The inclusion of live larvae was associated with modifications in the gut microbial community structure without compromising overall microbial diversity (Biasato *et al.*, 2019; Yadav and Jha, 2019). This selective modulation may be linked to specific components of *H. illucens* larvae, such as chitin, medium-chain fatty acids, and antimicrobial peptides (Cattaneo *et al.*, 2023), which are known to influence microbial populations. In line with previous studies on broilers fed insect-based diets (Biasato *et al.*, 2019), the enrichment of specific bacterial genera detected in larvae-fed chickens suggests a microbial shift driven by the dietary inclusion of *H. illucens*. These findings support the concept that insect-derived ingredients may serve not only as alternative protein sources but also as functional feed components with potential effects beyond their nutritional contribution (Grigore *et al.*, 2025; Hoffmans *et al.*, 2024; Pingali *et al.*, 2023; Gasco *et al.*, 2020).

A separate consideration concerns the increased abundance of *Staphylococcus* observed in the TRT group compared with the CTR group, which could represent a potential drawback associated with administration to larvae. Although members of this genus are commonly reported as components of the normal intestinal microbiota of chickens, *Staphylococcus* includes commensal species as well as pathogenic and opportunistic pathogenic strains. The presence of specific pathogenic strains may therefore result in infectious diseases in both humans and livestock animals (Syed *et al.*, 2020). However, identification based on partial or complete sequencing of the 16S rRNA gene provides limited taxonomic resolution for distinguishing closely related species and does not permit a reliable evaluation of pathogenic potential, which is often strain-dependent. Consequently, our data allow interpretation only at the

genus level and in terms of relative abundance. Regarding staphylococci, the relative abundance observed in both animal groups is consistent with values reported in the literature for healthy individuals (Ayala *et al.*, 2023). In the absence of clinical signs, these findings are therefore likely to have limited biological relevance.

The change in carcass fat FA composition observed in TRT chickens reflects the close relationship between dietary lipid sources and FA deposition in poultry tissues (Gasco *et al.*, 2019). When whole larvae are included in poultry diets, their lipid content must be carefully considered, as it influences the percentage of larvae that can be incorporated without causing an excessive increase in dietary fat (Benzertiha *et al.*, 2020; Gasco *et al.*, 2019). The characteristic lipid profile of *H. illucens* larvae, which is rich in medium-chain saturated FA, appears to be directly transferred to fat tissue (Benzertiha *et al.*, 2020). Although this change may have implications for the nutritional value of meat, it also offers opportunities for targeted modulation of meat composition through diet formulation (Cullere *et al.*, 2019). The increase in SFAs and the reduction in PUFA observed in the TRT group may influence the perceived nutritional value of poultry meat, which is traditionally considered favorable compared with red meat because of its higher PUFA content. However, these changes reflect the well-known relationship between dietary lipid composition and FA deposition in poultry tissues. As previously reported, the enrichment or reduction of PUFA in carcass fat largely depends on their dietary availability (Pinchasov and Nir, 1992). Moreover, medium-chain saturated FAs differ metabolically from long-chain SFAs, being more rapidly oxidized for energy. Therefore, the lipid profile of poultry meat can be optimized through appropriate dietary formulation strategies to maintain a balanced nutritional composition consistent with consumer expectations (Baéza *et al.*, 2021).

These results highlight the need to balance lipids derived from larvae with other dietary fat sources to achieve an optimal FA profile, particularly with respect to PUFA. Although a lower PUFA proportion may reduce oxidative susceptibility and potentially improve shelf life, it could also decrease the nutritional value of the lipid fraction. Therefore, combining insect-derived lipids with PUFA-rich sources appears necessary (Gasco *et al.*, 2019). From a sustainability perspective, the use of live *H. illucens* larvae represents a strategic approach in circular poultry meat production systems owing to the valorisation of organic byproducts (Pope *et al.*, 2023), reduction of dedicated feed crops (Allegretti *et al.*, 2018), and consequent reduction in the environmental footprint of poultry production (Bava *et al.*, 2019). However, further research is needed to optimize the inclusion levels, assess the long-term effects, and evaluate the feasibility in actual commercial farming.

In conclusion, this study shows that live *H. illucens* larvae can be included in chicken diets without compromising animal health and performance, with measurable effects on the gut microbiota and meat lipid composition of the birds. These results provide a basis for future research on precision poultry nutrition using *H. illucens* live larvae and also provide findings for sustainable development of the poultry sector.

Author contributions

F.F. formal analysis, writing (original draft, review and editing), visualization, data curation; R.M.C. investigation, writing (review and editing); S.S. investigation, formal analysis; C.M.L. investigation, formal analysis; P.V. investigation, formal analysis; N. M. investigation, resources; P.A. conceptualization, project administration, writing (review and editing), validation; M.E. conceptualization, project administration, writing – review and editing, funding acquisition.

Conflict of interest

The authors declare that they have no competing financial interests or personal relationships that may have influenced the work reported in this study.

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TABLE A1 Analytical composition and additives of the commercial complementary feed (NP32SS-Nucleo Polli 2P Oscar) used in the experimental trials

Nutrient composition[1]	
Crude Protein (%)	40.0
Crude Fat (%)	5.7
Crude Fibre (%)	3.8
Ash (%)	11.5
Sodium (%)	0.3
Methionine (%)	0.8
Lysine (%)	2.1
Additives[2]	
Vitamin A (IU)	12 800
Vitamin D3 (IU)	4800
Vitamin E (mg)	64
Vitamin K3 (mg)	4.0
Vitamin B2 (mg)	8.8
Vitamin B12 (mg)	0.030
Niacin (mg)	20
Folic acid (mg)	1.6
Biotin (mg)	0.24
Choline chloride (mg)	1650
Iron (mg)	48
Copper (mg)	20
Zinc (mg)	128
Manganese (mg)	144
Iodine (mg)	1.9
Selenium (mg)	0.4
Sodium propionate (mg)	2499
Lutein (mg)	161
Zeaanthin (mg)	11.5
Canthaxanthin (mg)	10.0

1 Values declared by the manufacturer and expressed per g/100 g of feed.

2 Values declared by the manufacturer and expressed per kg of feed.