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Live black soldier fly larvae as environmental enrichment for native chickens: implications for bird performance, welfare, and excreta microbiota

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ABSTRACT

Dietary live insect larvae were recently proposed for use in laying hens and broiler—intensive chicken farming as an innovative form of environmental enrichment, but their use in native dual-purpose chickens has never been investigated. This study aims to evaluate the effects of live black soldier fly (**BSF**) larvae as environmental enrichment in two autochthonous dual-purpose chicken breeds, namely Bionda Piemontese (**BP**) and Bianca di Saluzzo (**BS**), in terms of bird performance, behaviour, integument status, excreta corticosterone metabolites (**ECMs**), and microbiota analyses. A total of 90 BP and 90 BS hens aged 308 days old were randomly distributed between two treatment groups (three replicates/group/breed, 15 hens/replicate). For the following 90 days, the control group (**C**) was fed a commercial feed only, whereas the BSF group was fed the commercial diet plus BSF live larvae calculated at 6% of the expected daily feed intake (DFI). Larva ingestion time, bird performance, integument scores, and behavioural observations were assessed at regular intervals, and excreta samples were collected to evaluate ECM and microbiota. The larva ingestion time became faster over the course of the experimental trial ($P < 0.001$). The DFI of BSF-fed hens was lower than that of C hens independently of breed ($P < 0.001$), whereas only in the BS hens, the live weight of the BSF-fed group was greater than that of the C group ($P < 0.01$). The BSF-fed BP hens showed a higher laying rate and feed conversion ratio compared with BSF-BS ($P < 0.05$). Better total integument scores were observed in BSF-fed BP hens compared with C-birds ($P < 0.05$). The BSF-fed hens displayed higher frequencies of preening, trotting, and wing flapping than C, as well as a lower incidence of severe feather pecking ($P < 0.05$). An increase in allopreening was only identified in BSF-fed BS hens with respect to the C hens ($P < 0.001$). No differences in ECM and faecal microbiota were observed between treatment groups. In conclusion, the administration of BSF live larvae as environmental enrichment has the potential to positively influence the welfare of both BP and BS chickens, by enhancing the frequency of positive behaviours whilst reducing severe feather pecking, without affecting their excreta microbiota. BSF larva administration also has the potential to improve the productive performance and the plumage status of the BP breed.

Implications

The study underscores the potential of using live black soldier fly larvae for environmental enrichment in native, slow-growing dual-purpose poultry breeds—a practice well-studied in commercial farming but largely overlooked in local breeds. The supplementation of larvae not only enhances positive behaviours but also reduces feather pecking, thus indicating its value in improving animal welfare. Specialised farms that focus on local breeds are particularly suited to implement this practice, given their capacity to manage the specific requirements of larvae supplementation, such as biomass handling. Future research should aim to refine these methods and assess their broader impacts on welfare and farm sustainability.

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Introduction

As a consequence of the recent advances in poultry genetics, nutrition, housing, and management strategies, the poultry industry has become a leading supplier of efficient, high-quality animal protein on a worldwide basis (Kover, 2023). That said, consumers are becoming increasingly concerned about animal welfare issues, which can influence their purchasing choices and, in turn, the environmental sustainability of intensive farming systems (broilers and laying hens). In particular, ever more consumers and citizens are calling for ethical production systems, and many are refusing to buy products that do not respect their animal welfare concerns, despite a relatively low level of knowledge about farming and animal welfare issues (Alonso et al., 2020). In line with this strand of thought, consumer attention has recently been directed towards alternative poultry production systems, such as slow-growing chicken breeds and free-range hens. In this context, the farming of dual-purpose and native chicken breeds offers the possibility for some appealing niche productions (Tiemann et al., 2020). Dual-purpose breeds are characterised by their suitability in terms of both egg and meat production, and they tend not to be not associated with the challenges typical of highly specialised lines (Becker et al., 2023). Native chicken breeds, on the other hand, adapt easily to different local housing and environmental conditions, making them well suited for extensive farming systems (Tiemann et al., 2020). Italy is one of the cradle countries valorising native chickens, being home to 53 recognised local chicken breeds (Zanon and Sabbioni, 2001), numerous conservation programmes, and a National Registry that currently includes 22 local breeds (Castillo et al., 2021). Of these breeds, Bionda Piemontese (**BP**) – characterised by its blond plumage and a black tail – and Bianca di Saluzzo (**BS**) – completely white – are receiving increasing attention from the scientific community (Castillo et al., 2024; Franzoni et al, 2021; Soglia et al., 2021). Both are dual-purpose breeds originating from the Piedmont region (Northwest Italy) that thrive in organic and free-range rearing systems (Ferrante et al., 2005; Soglia et al., 2020). The BP and BS breeds are reared for both meat and egg production, but the research to date has mainly focused on the evaluation of their carcass yields and meat quality (Bongiorno et al., 2022a), making their potential as egg-laying strains largely undocumented.

A decade of scientific research has already highlighted the huge potential of using insects to improve the sustainability of the poultry supply chain. The industry can presently rely on two different lines of insect production: (1) larvae can either be processed into meals or the fats extracted to supply poultry diets with protein and energy sources, respectively; (2) live or dehydrated larvae can be fed to the chickens whole, thus also exploiting their potential as a form of environmental enrichment (Schiavone and Castillo, 2024). Environmental enrichment is generally defined as any modification to the environment of captive animals which permits the behavioural repertoire of the animals to improve and, in turn, the animals' biological functions (Newberry, 1995). Since live, moving insects form part of the chicken's natural diet, the scientific community has recently advanced the idea of using live insect larvae as an alternative form of environmental enrichment for the main poultry strains (Bongiorno et al., 2022b; Ipema et al., 2020; Star et al., 2020; Veldkamp and van Niekerk, 2019; Gariglio et al., 2023). So far, the provision of live black soldier fly (**BSF**) larva, alone or alongside yellow mealworm, has been reported to have a positive influence on turkey, broiler, laying hen, medium-growing chicken, and Muscovy duck performance (Veldkamp and van Niekerk, 2019; Bellezza Oddon et al., 2021; Tahamtani et al., 2021; Ipema et al., 2022), behaviour (Pichova et al., 2016; Veldkamp and van Niekerk, 2019; Ipema et al., 2020 and 2022; Star et al., 2020; Biasato et al., 2022; Gariglio et al., 2023), feather condition (Star et al., 2020), excreta corticosterone metabolites (Gariglio et al., 2023), and caecal microbiota (Huang et al., 2024), without impairing animal health status (Bellezza Oddon et al., 2021; Bongiorno et al., 2022b). However, the potential of using live insect larvae in native dual-purpose breeds has yet to be explored. Doing so would allow us to: (1) investigate the possibility of making further improvements to the welfare conditions of birds that already respond well to the novel environmental inputs, and (2) help create a niche production with the potential of being further valorised, as purebred dual-purpose breeds might provide small-scale farmers with a means to gain independence from commercial breeding companies and to generate extensive production systems (Meuser et al., 2021). The hypothesis posits that live larvae, as a potential component of commercial products, may effectively address the outlined issues by improving the welfare conditions of birds and, subsequently, enhance production performance. Therefore, the aim of the present study was to investigate the effects of BSF live larvae as environmental enrichment for BP and BS laying hens, providing a multiperspective view on bird performance, welfare (behaviour, integument status, and excreta corticosterone analyses), and excreta microbiota.

Material and methods

Birds and husbandry

The experimental trial was carried out in the poultry facility of the Avian Conservation Centre for the Valorization of Local Genetic GENetic Resources (CoVaGEN) of the University of Turin (Italy) (44°50′58″ N and 7°43′13″ E), officially recognised by the Italian Ministry of Agriculture and Forestry Policies in 2016. The experimental protocol (Prot. No. 814715) was approved by the Bioethical Committee of the University of Turin (Italy). The trial lasted 90 days and was conducted in BP and BS hens simultaneously. A total of 180 adult (308 days old) laying hens (90 belonging to the BP breed and 90 to the BS breed) were randomly assigned to 12 pens (15 hens per pen). The hens were allocated in order that the mean initial live weight was the same for the two dietary treatments (C: 2405 ± 40.2 g (SEM); BSF: 2433 ± 41.8 g). Each pen, covered with rice hulls as litter, measured 3.50 m × 2.00 m and was equipped with feeders, bell drinkers, and nests. The pens contained no forms of environmental enrichment (such as perches), but had access to an outdoor paddock area (4.00 m × 2.00 m). Chickens were exposed to light between 1436 and 1536 h, and the environmental temperature ranged from 8 to 37 °C during the trial, which was conducted from May to August. All the hens had been since their hatching under the same conditions and previously vaccinated against Newcastle, Marek, and Gumboro diseases, as well as coccidiosis.

Experimental treatments and chemical analyses

Each pen of each breed was randomly assigned to one of the two experimental treatments (three replicate pens/treatment, 15 hens/pen) as follows: (i) control group (**C**): fed commercial feed for adult laying hens, and (ii), BSF: fed the C diet + BSF live larvae calculated on the fresh matter basis as 6% of the expected daily feed intake (**DFI**) (120 g/bird (7.2 g of live larvae)) (NRC, 1994). The commercial diet (provided by Fratelli Borello S.p.A., Bra – CN, Italy) included wheat meal, soybean meal, sunflower meal, calcium carbonate, dicalcium phosphate, cane molasses, sodium chloride, sodium bicarbonate, and a vitamin-mineral premix (DM, 87.00%; CP, 16.00% as is; ether extract (**EE**), 2.70% as is; crude fibre (**CF**), 3.60% as is; ash, 12.60% as is; calcium, 4.06% as is; phosphorus,

0.43% as is; sodium, 0.15% as is; lysine, 0.80% as is; methionine, 0.30% as is; apparent metabolisable energy corrected for zero nitrogen retention, 12.50 MJ/kg as is). Feed and water were distributed *ad libitum* in both treatments. The daily quantity of larvae was placed in two plates inside each pen, distributed at 1100 h (6 days/week). Every day, the time spent by the hens eating the larvae was recorded through continuous observation, according to Bellezza Oddon et al. (2021). The recorded values (seconds) were averaged for different periods (days 0–30, 31–60, and 61–90) to evaluate the adaptation of the birds over the course of the experimental trial. The quantity of BSF larvae needed to satisfy 6% of the DFI was sent by Entomics Biosystems Limited (Cambridge, United Kingdom) on a weekly basis. Upon their arrival at the poultry facility, larvae were preserved according to the guidelines reported in Bellezza Oddon et al. (2021) until the time of administration to the animals. Briefly, BSF larvae were kept in a climatic chamber at 16 °C to trigger the diapause mechanism and allow their preservation until administration to the hens. Samples of larvae were periodically collected, killed by freezing (−20 °C), and stored at the same temperature until the time of chemical analysis.

Larvae arrived at the poultry centre on a weekly basis throughout the experimental trial, and a sample was immediately stored at −20 °C for subsequent chemical analyses. All the samples were subsequently freeze-dried and ground~~ed~~ using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland). Samples were analysed for: dry matter (DM, method number 943.01), ash (method number 924.05), CP (method number 984.13; N-P conversion factor = 4.67; Janssen et al., 2017), and ether extract (EE, method number 2003.05) according to International AOAC (DM, ash, and CP (AOAC, 2000); EE (AOAC, 2003)). The gross energy content was determined using an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany).

Bird performance and integument score

All the hens were individually labelled with a wing mark at the beginning of the experimental trial. Health status and mortality were monitored daily across the experimental period. The BW of each animal was recorded at the beginning, middle, and end of the experimental trial (days 0, 45, and 90) using electronic scales (Sartorius – Signum®, Gottinga, Germany). The DFI was recorded every 30 days (on days 30, 60, and 90). Egg weight (**EW**) was recorded daily and expressed as an average value every 30 days (days 30, 60, and 90 of the experiment). The laying rate (**LR**) was obtained by multiplying the egg production (**EP**), calculated by dividing the number of eggs collected each day by the number of hens on that day) by 100. The feed conversion ratio (**FCR**) was calculated as the ratio between the average DFI and the average egg mass (obtained by multiplying the average EP by the average EW). The FCR calculation included the DM intake derived from the commercial feed (C group) and that derived from the commercial feed plus BSF supplementation calculated on a DM basis (1.83 g/day/chicken consumed by the BSF group). The average values of EP, LR, and FCR were calculated every 30 days (on days 30, 60 and 90 of the experiment).

At the beginning, middle, and end of the experimental trial (days 0, 45, and 90 of the experiment), each hen was assigned an integument score – always by the same operator – for neck, breast, cloaca/vent, back, wings, and tail, and bumble foot lesions: the higher the score, the better the integument status. This system permits the assessment of both individual body regions (scores: 1, 2, 3 or 4) and the whole body (scores: 6–24) (Tauson et al., 2015).

Behavioural observations

The behavioural observations were carried out by means of video recordings. All the pens were filmed for 10 min before the administration of the live BSF larvae at the beginning of the experimental trial and every 30 days (days 0, 30, 60, and 90). The videos were then analysed in continuous mode by Behavioural Observation Research Interactive Software (BORIS; Friard and Gamba, 2016), following the procedures reported in Biasato et al. (2022). As reported in Table 1, the behaviours were divided into two categories: the frequency behaviours, which were assessed on all the birds in each replicate, and the duration behaviour, which were determined on four animals per pen.

Analysis of excreta corticosterone metabolites

At the beginning of the experimental trial (day 0) and every 30 days (days 30, 60, and 90), fresh excreta samples were collected directly from the ground over the course of the morning (0800–1000 h) to obtain three pools/treatment/breed. After collection, the excreta samples were immediately frozen and stored at −20 °C until corticosterone analysis and processed according to Biasato et al. (2022).

Excreta microbiota characterisation

At the beginning, middle, and end of the experimental trial (days 0, 45, and 90), all the hens were removed from their pens and individually housed in wire-mesh cages (100 cm × 50 cm) for 30 min to collect fresh excreta samples that were immediately frozen at −80 °C until DNA extraction and 16S rRNA amplicon target sequencing. DNA was extracted from the excreta samples using the RNeasy Power Microbiome KIT (Qiagen, Milan, Italy). Following

Table 1
Description of the laying hen ethogram (frequency and duration of behaviours) considered in the present study.

Behaviours	Definition
Frequency	
Scratching	Scraping of the litter with the claws (Ipema et al., 2020)
Preening	Grooming of own feathers with beak (Ipema et al., 2020)
Severe feather pecking	Forceful pecks that result in feathers being pulled out, accompanied by a reaction of the recipient bird (Rieke et al., 2021)
Chasing	One hen chasing another, with fast running, no vocalisations, no hopping and no wing flapping (Sokołowicz et al., 2020)
Allopreening	Social grooming (Kenny et al., 2017)
Duration	
Walking	Taking one or more steps (Webster and Hurnik, 1990)
Standing still	Standing on the feet with extended legs (Webster and Hurnik, 1990)
Ground pecking	Pecking at the litter with the head in a position lower than the rump (van Hierden et al., 2002)
Lying down	Sitting position (Webster and Hurnik, 1990)

Source: Biasato et al. (2022).

treatment with RNase (Illumina Inc, San Diego, CA), DNA was quantified using the Qubit assay and standardised at 5 ng/μl. The DNA extracted from the excreta samples was used to amplify the V3-V4 region of the 16S rRNA gene (Klindworth et al., 2013). PCR products were purified and tagged according to the Illumina metagenomic standard procedure (Illumina Inc., San Diego, CA). Sequencing was performed on a MiSeq Illumina instrument with V3 chemistry and generated 250 bp paired-end reads according to the manufacturer's instructions.

Statistical analysis and bioinformatics

Statistical analysis of the collected data was performed using IBM SPSS Statistics V27.0.0 software (IBM, Armonk, NY, USA). The pen was considered the experimental unit for all parameters considered. Shapiro-Wilk's test established the normal or non-normal distribution of the data and residuals. The larva intake times and the laying performance (which displayed normally distributed residuals) were analysed by fitting a general linear mixed model. The larva intake time (minutes) depended on three fixed factors (breed, time, and their interaction), whereas the laying performance depended on five fixed factors (diet, breed, time, the interaction between diet and breed, and the interaction between diet and time). In all the above-mentioned models, the replicate was included as a random effect to account for repeated measurements on the same pen. The interactions between the levels of the fixed factors were evaluated by means of pairwise contrasts. The integument scores, the behavioural data, and the corticosterone concentrations (which displayed non-normally distributed residuals) were analysed by fitting a generalised linear mixed model. The integument scores depended on linear predictors (diet, breed, time, the interaction between diet and breed, and the interaction between diet and time) through a negative binomial response *P* distribution with a non−linear link function (log), whereas the behaviours or the corticosterone concentrations depended on linear predictors (diet, breed, time, the interaction between diet and breed, and the interaction between diet and time) through a gamma *P* distribution with a non−linear link function (log). The replicate was included as a random effect to account for repeated measurements on the same pen (behavioural and corticosterone data) or bird (integument scores), and the interactions between the levels of the fixed factors were also evaluated by means of pairwise contrasts. The results were expressed as least square means plus the SE of the mean. *P*-values $\leq$ 0.05 were considered statistically significant.

To analyse excreta microbiota findings, paired-end reads were first merged using FLASH software by setting default parameters (Magoč and Salzberg, 2011). Joint reads were further filtered for chimeric sequences and for quality (at Phred < Q20) using QIIME 2 software and the dada2 denoise-paired method (Callahan et al., 2017) to obtain the Amplicon sequence variants. Taxonomy of amplicon sequence variants was obtained through the QIIME feature-classifier against the Greengenes database. The amplicon sequence variants table was then rarefied at the lowest number of sequences, and it displays the highest taxonomic resolution. Alpha and beta diversity indices were calculated using the diversity function of the QIIME 2 and analysed using the pairwise Wilcoxon rank sum test to assess for differences between the experimental treatments. Weighted UniFrac distance matrices and the amplicon sequence variants table were used to perform ADONIS and ANOSIM statistical tests in R environment. A generalised linear model was applied to test the importance of continuous or discrete variables (such as diet, breed, time, and their corresponding interactions) on the amplicon sequence variants relative abundance. *P*-values were adjusted for multiple testing (Bonferroni correction), and a false discovery rate (**FDR**) < 0.05 was considered as significant.

Results

Larvae chemical composition and ingestion times

The proximate composition of BSF larvae was as follows (on average): DM, 255 g/kg as is; CP, 368 g/kg DM; EE, 114 g/kg DM; ash, 147 g/kg DM; AME, 8.36 MJ/kg DM).

Based on the chosen BSF supplementation level (kept constant throughout the experimental trial), each bird consumed approximately 1.82 g of DM, 0.67 g of CP (on DM), 0.21 g of EE (on DM) and 0.016 MJ/Kg AME (on DM), on a daily basis. The average ingestion time was similar in the two breeds (BP, 3.27 ± 0.28 min; BS, 2.50 ± 0.28 min), and there was no interaction between diet and trial period. However, in both breeds, the larva consumption time was faster in the latter two periods (days 31–60 and 61–90) compared with the initial period (days 0–30) ($P < 0.001$, Fig. 1).

Bird performance and integument score

Table 2 and Fig. 2 summarise the performances of the two breeds according to dietary treatment. The BW of the hens depended on both the diet and the interaction between diet and breed ($P = 0.003$ and $P = 0.020$). Specifically, the BW of BS laying hens fed the live BSF larvae was only greater than that of the C-fed BS hens ($P = 0.016$, Fig. 2A). The LR depended on the interaction between live BSF larva administration and breed only ($P = 0.046$). In particular, the BP breed fed the live BSF larvae displayed a higher LR than BS laying hens fed BSF larvae ($P = 0.045$, Fig. 2B). By contrast, EW depended on both the breed and the time ($P = 0.009$ and $P < 0.001$, respectively). In detail, independently of the provision of live BSF larvae, the BP laying hens produced heavier eggs than the BS breed ($P = 0.009$), with an increase in EW also being identified on day 90 with respect to days 30 and 60 ($P < 0.001$). The DFI depended on almost all the considered variables ($P < 0.001$). In particular, and independently of breed, the DFI− of BSF-fed laying hens was lower than that of the C group across the entire experimental trial ($P < 0.001$, Fig. 2C). Independently of the administration of live BSF larvae, the DFI of the BP breed was lower than that of BS laying hens ($P < 0.001$). Lastly, the FCR

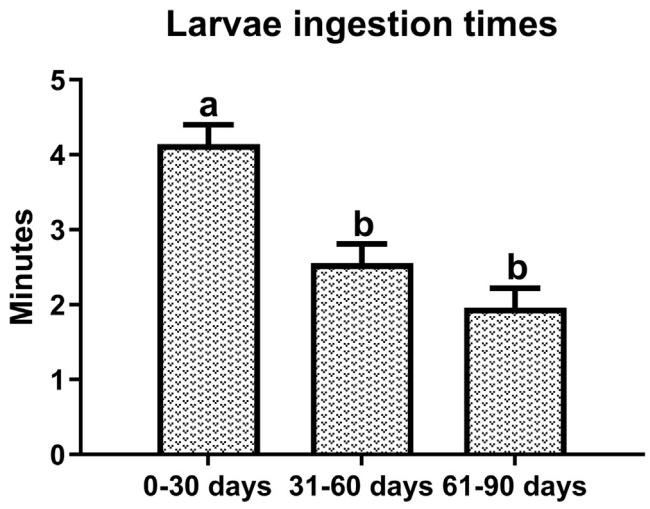

Fig. 1. Time spent by the laying hens on eating the live black soldier fly larvae. Graph bars with different superscript letters indicate significant differences ($P < 0.05$) between the trial periods.

Table 2
Laying performance of the laying hens according to diet, breed, time, and their corresponding interactions.

Item	Enrichment (E)		Breed (B)		Time (T)[1]			SEM			*P*-value				
	C	BSF	BP	BS	T1	T2	T3	E	B	T	E	B	T	E × B	E × T
BW, g	2 359	2 456	2 424	2 391	2 389	2 409	2 424	22.85	22.84	27.95	0.003	0.310	0.669	0.020	0.926
LR, %	56.0	54.0	54.8	55.2	58.0	54.9	52.1	2.66	2.98	2.29	0.622	0.928	0.116	0.046	0.624
EW, g	61.2	60.0	61.7	59.5	60.1^{a}	60.4^{a}	61.4^{b}	0.71	0.67	0.53	0.274	0.009	<0.001	0.524	0.616
DFI, g	108	105	106	107	103^{a}	106^{b}	111^{c}	0.17	0.16	0.19	<0.001	<0.001	<0.001	0.072	<0.001
FCR, n	3.30	3.40	3.29	3.41	3.12	3.32	3.62	0.21	0.20	0.18	0.760	0.649	0.054	0.020	0.426

Abbreviations: C = control group; BSF = C diet + live black soldier fly larvae. BP = Bionda Piemontese; BS = Bianca di Saluzzo; LR = laying rate; EW = egg weight; DFI = daily feed intake; FCR = feed conversion ratio.
Experimental unit: pen (n = 3). Applied statistical model: general linear mixed model (GLMM), with five fixed factors (diet, breed, time, interaction between diet and breed, and interaction between diet and time), replicate as a random effect, and pairwise comparisons to compare means. Statistical software: IBM SPSS Statistics V27.0.0 (IBM, Armonk, NY, USA).
[1] BW: T1 = day 0; T2 = day 45; T3 = day 90; LR, EW, and FCR: T1 = day 30; T2 = day 60; T3 = day 90; DFI: T1 = days 1–30; T2 = days 30–60; T3 = days 60–90. Means with different superscript letters differ significantly among the effect levels ($P < 0.05$).

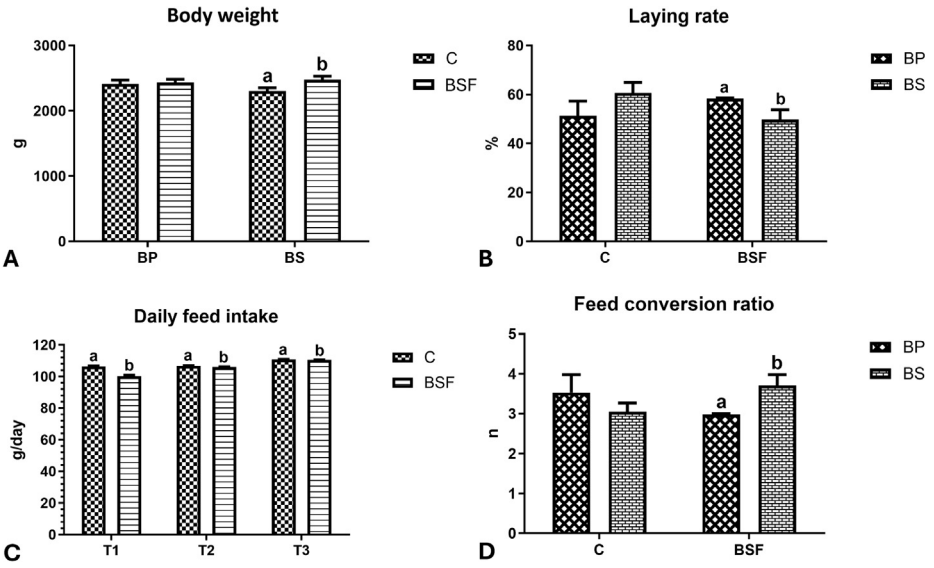

Fig. 2. Laying hens performances. (**A**) BW. (**B**) Laying rate (LR). (**C**) Daily feed intake (DFI). (**D**) Feed conversion ratio (FCR). Graph bars with different superscript letters indicate significant differences ($P < 0.05$). T1 = days 1–30; T2 = days 31–60; T3 = days 61–90; C = control group; BSF = C diet + live black soldier fly larvae; BP = Bionda Piemontese; BS = Bianca di Saluzzo.

Table 3
Integument scores of the laying hens depending on diet, breed, time, and their corresponding interactions.

Item	Enrichment (E)		Breed (B)		Time (T)			SEM			*P*-value				
	C	BSF	BP	BS	T1	T2	T3	E	B	T	E	B	T	E × B	E × T
Total scores, n	21.4	22.1	23.3	20.2	22.7^{a}	21.6^{b}	21.0^{c}	0.26	0.26	0.22	0.093	<0.001	<0.001	0.032	0.207

Abbreviations: C = control group; BSF = C diet + live black soldier fly larvae; BP = Bionda Piemontese; BS = Bianca di Saluzzo; T1 = day 0; T2 = day 45; T3 = day 90.
Means with different superscript letters differ significantly among the effect levels ($P < 0.05$).
Experimental unit: pen (n = 3). Applied statistical model: generalised linear mixed model (GLMM, negative binomial response *P* distribution with a non−linear link function (log)), with five fixed factors (diet, breed, time, interaction between diet and breed, and interaction between diet and time), replicate as a random effect, and pairwise comparisons to compare means. Statistical software: IBM SPSS Statistics V27.0.0 (IBM, Armonk, NY, USA).

depended on the interaction between the live BSF larva administration and the breed only ($P = 0.020$). In particular, the BP breed administered live BSF larvae displayed a lower FCR when compared with the BSF-fed BS laying hens ($P = 0.009$, Fig. 2D).

Table 3 and Fig. 3 report the integument scores for four bird groups. The total integument scores depended on breed ($P < 0.001$), time ($P < 0.001$), and interaction between diet and breed ($P = 0.032$). In particular, the total integument scores for the BP breed fed the live BSF larvae were better than those for the C group ($P = 0.037$; Fig. 3). Specifically, the numerical values for the area of the integument in the C and BSF groups were as follows: 3.3 and 3.5 for the neck, 2.6 and 3.0 for the cloaca, 2.2 and 2.1 for the back, 3.4 and 3.5 for the wings, 2.5 and 3.0 for the tail, and 3.3 for the breast in both groups. Furthermore, independently of live BSF larva administration, total integument scores progressively worsened over the course of the experimental trial ($P < 0.001$).

Behavioural observations

The frequency and duration of the laying hen behaviours are summarised in Table 4. Chasing behaviour was not affected by

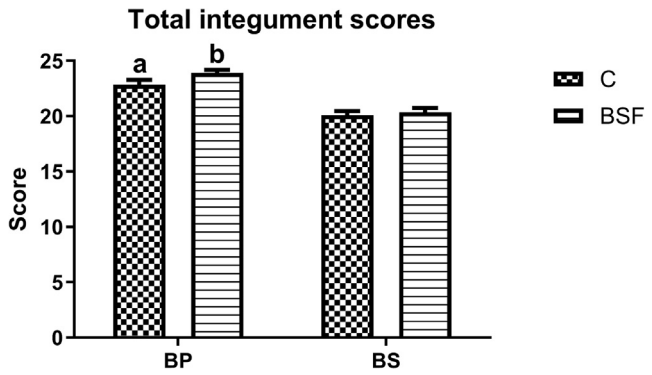

Fig. 3. Plumage status of the laying hens. Total integument scores. Graph bars with different superscript letters indicate significant differences ($P < 0.05$) between the experimental treatments within each breed. C = control group; BSF = C diet + live black soldier fly larvae; BP = Bionda Piemontese; BS = Bianca di Saluzzo.

the treatments. By contrast, preening and severe feather pecking were influenced by live BSF larva administration ($P < 0.001$ (preening), $P = 0.015$ (severe feather pecking)). Specifically, the BSF-fed laying hens preened more frequently than the C group, and exhibited less frequent severe feather pecking ($P < 0.001$ (preening) and $P = 0.015$ (severe feather pecking)). By contrast, the frequency of allopreening was affected by diet, breed, and the interaction between diet and breed ($P = 0.002$ (breed) and $P < 0.001$ (diet and diet × breed)). In particular, allopreening frequency was greater in the BS breed fed the live BSF larvae with respect to the C-fed BS laying hens ($P < 0.001$, Fig. 4). Regarding the duration of behaviours, only the time spent walking depended on the administration of live BSF larvae ($P = 0.015$), with the BSF-fed laying hens spending more time walking than the C group ($P = 0.015$). The time spent standing still, ground pecking, and laying down were all influenced by the experimental period and/or breed (time: standing still ($P = 0.002$), ground pecking ($P < 0.001$) and laying down ($P = 0.012$); breed: laying down ($P < 0.001$)). In detail, independently of larva administration, laying hens spent more time standing still on days 30, 60, and 90 than on day 0 ($P = 0.002$), whereas less ground pecking ($P < 0.001$) and laying down behaviour ($P = 0.012$) were observed on days 60 and 30, respectively, compared with on day 0. Furthermore, the BP breed spent less time laying down than the BS hens ($P < 0.001$).

Excreta corticosterone analysis

The excreta corticosterone metabolites (**ECM**) of the laying hens (Table 4) depended on both breed and experimental period ($P = 0.022$ and $P < 0.001$, respectively). In particular, independently of the administration of live BSF larvae, the ECM was higher in the BS breed than in the BP hens ($P = 0.020$). Furthermore, a progressive increase in ECM was observed from day 0 to day 60 ($P < 0.001$).

Excreta microbiota characterisation

Sequencing produced a total of 779 584 reads. After filtering for quality, 532 148 reads were used for downstream analysis, with an average of 14 604 ± 5 261 reads per sample. Rarefaction analysis and the estimated sample coverage indicated a satisfactory coverage of all samples (median estimated sample coverage value of 92%). Neither diet nor breed influenced the alpha-diversity (**FDR** > 0.05). By contrast, the alpha-diversity of the excreta microbiota depended on the experimental period, showing a reduced value on day 45 followed by an increase on day 90 (FDR < 0.05, Fig. 5). Analogous differences were also highlighted through ADONIS and ANOSIM statistical tests based on weighted UniFrac distance matrices ($P < 0.001$). Indeed, principal component analysis (PCoA) revealed a clear and progressive separation of excreta microbiota as a function of the time (Fig. 6).

Fig. 7 summarises the evolution of the excreta microbiota over the course of the experimental trial. In particular, the main phyla represented (Fig. 7A) were Firmicutes (59.20, 83.57, and 79.41% of the relative abundance on days 0, 45, and 90, respectively), Bacteroidetes (24.60, 4.26, and 9.20%) and Actinobacteria (8.56, 5.85, and 7.02%). As far as the genera are concerned (Fig. 7B), the main microbiota was dominated by *Lactobacillus* (17.70, 34.34, and 38.55% of the relative abundance at days 0, 45, and 90, respectively), *Turicibacter* (9.95, 18.22, and 8.76%), Bacteroides (9.49,

Table 4
Frequency, duration behaviours, and excreta corticosterone metabolites of the laying hens according to diet, breed, time, and their corresponding interactions.

Item	Enrichment (E)		Breed (B)		Time (T)				SEM			*P*-value				
	C	BSF	BP	BS	T0	T1	T2	T3	E	B	T	E	B	T	E × B	E × T
Frequency behaviours																
Scratching, n	7.42	4.46	5.79	6.08	10.08	8.25	3.00	2.42	1.48	1.48	1.68	0.174	0.893	0.053	0.200	0.054
Preening, n	3.13	5.08	3.71	4.50	4.75	3.75	3.25	4.67	0.33	0.33	0.82	<0.001	0.107	0.134	0.671	0.621
Severe feather pecking, n	1.50	0.50	0.88	1.13	1.58	0.92	0.58	0.92	0.24	0.28	0.36	0.015	0.542	0.473	0.839	0.601
Chasing, n	1.00	0.50	0.42	1.08	1.08	1.08	0.33	0.50	0.28	0.25	0.31	0.219	0.101	0.087	0.682	0.285
Allopreening, n	0.00	0.29	0.04	0.25	0.00	0.25	0.17	0.17	0.04	0.05	0.13	<0.001	0.002	0.100	0.002	0.100
Duration behaviours																
Walking, %	15.6	22.3	18.6	19.4	17.3	20.3	21.2	17.1	1.93	1.89	2.78	0.015	0.773	0.767	0.607	0.858
Standing still, %	40.5	38.0	39.2	39.3	31.5^{a}	42.3^{b}	42.9^{b}	40.3^{b}	2.46	2.35	3.06	0.473	0.981	0.002	0.464	0.111
Ground pecking, %	30.5	32.1	32.2	30.4	35.7^{a}	32.2^{ab}	24.6^{b}	32.7^{ab}	3.09	2.71	3.73	0.720	0.671	<0.001	0.812	0.707
Laying down, %	9.86	6.78	3.64	13.0	13.2^{a}	2.45^{b}	8.56^{a}	9.12^{a}	1.33	1.30	2.84	0.123	<0.001	0.012	0.831	0.247
Excreta																
ECM, ng/g	3 537	3 578	3 450	3 668	$2\ 565^{a}$	$3\ 191^{b}$	$4\ 506^{c}$	$4\ 342^{c}$	65.33	63.77	78.73	0.660	0.022	<0.001	0.900	0.170

Abbreviations: C = control group; BSF = C diet + live black soldier fly larvae. BP = Bionda Piemontese; BS = Bianca di Saluzzo; T0 = day 0; T1 = day 30; T2 = day 60; T3 = day 90; ECM = excreta corticosterone metabolites.
Means with different superscript letters differ significantly among the effect levels ($P < 0.05$).
Experimental unit: pen (n = 3). Applied statistical model: generalised linear mixed model (GLMM, gamma *P* distribution with a non−linear link function (log)), with five fixed factors (diet, breed, time, interaction between diet and breed, and interaction between diet and time), replicate as a random effect, and pairwise comparisons to compare means. Statistical software: IBM SPSS Statistics V27.0.0 (IBM, Armonk, NY, USA).

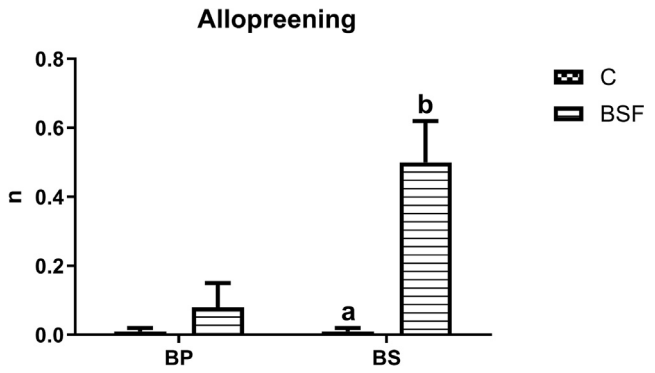

Fig. 4. Allopreening of the laying hens. Graph bars with different superscript letters indicate significant differences ($P < 0.05$) between the experimental treatments within each breed. C = control group; BSF = C diet + live black soldier fly larvae; BP = Bionda Piemontese; BS = Bianca di Saluzzo.

1.95, and 4.38%), *Clostridium* (9.13, 10.67, and 12.44%), and *Enterococcus* (3.69 12.37, and 8.44%). The administration of the live BSF larvae did not influence the excreta microbiota ($P > 0.05$), but it did depend on both the time and breed ($P < 0.05$). Specifically, the relative abundances of Bacteroidetes, Fusobacteria, and Verrucomicrobia were lower on day 45 (FDR < 0.05) with respect to prior levels, whereas Firmicutes were higher on days 45 and 90 (FDR < 0.01). Concerning changes in genera, *Lactobacillus* and *Streptococcus* were higher on day 45 (FDR < 0.01 and FDR < 0.05, respectively), with higher relative abundances also identified in the BP hens compared with the BS hens (FDR < 0.01 and FDR < 0.05, respectively). Furthermore, the amplicon sequence variants for Fusobacteriaceae and *Blautia* were reduced on day 45 (FDR < 0.01 and FDR < 0.05, respectively), with a greater relative abundance of *Turicibacter* on day 45 compared with on days 0 and 90 (FDR < 0.05).

Discussion

The present study revealed that the administration of live BSF larvae as a means to enrich the environment of dual-purpose local Italian chicken breeds was capable of modulating bird performance and welfare on a multitude of levels, and revealed a remarkable influence of both breed and time on the evaluated parameters.

Larva ingestion times

The time taken for the laying hens to consume the live BSF larvae was faster in the final 60 days than in the first 30 days of the 90-day experimental trial. It is reasonable to conclude that this reflects the natural ability of these hens to adapt to novel stimuli in their rearing environments, as already suggested by Bellezza Oddon et al. (2021). Furthermore, we observed similar consumption times in the BP and the BS hens, indicating a comparable aptitude to eat live larvae.

Bird performance and integument score

As far as bird performance is concerned, the BSF-fed laying hens showed a reduction in DFI at each of the considered experimental time-points. This may be related to the high nutritional quality of the BSF larvae, which can supplement the protein and fat content in the basal feed (Star et al., 2020). Furthermore, considering that the live BSF larvae were used here as a source of environmental enrichment, i.e. as a supplement to a complete diet and not as a feed ingredient required to meet the birds' dietary needs, the observed reduction in the mash feed intake was reasonable. Another interesting aspect to underline is that the difference in the DFI was particularly evident in the first period of the trial (1–30 days), with a progressive attenuation of this difference until the end of the experimental trial. This provides further confirmation of the progressive ability of the birds to adapt, in terms of their

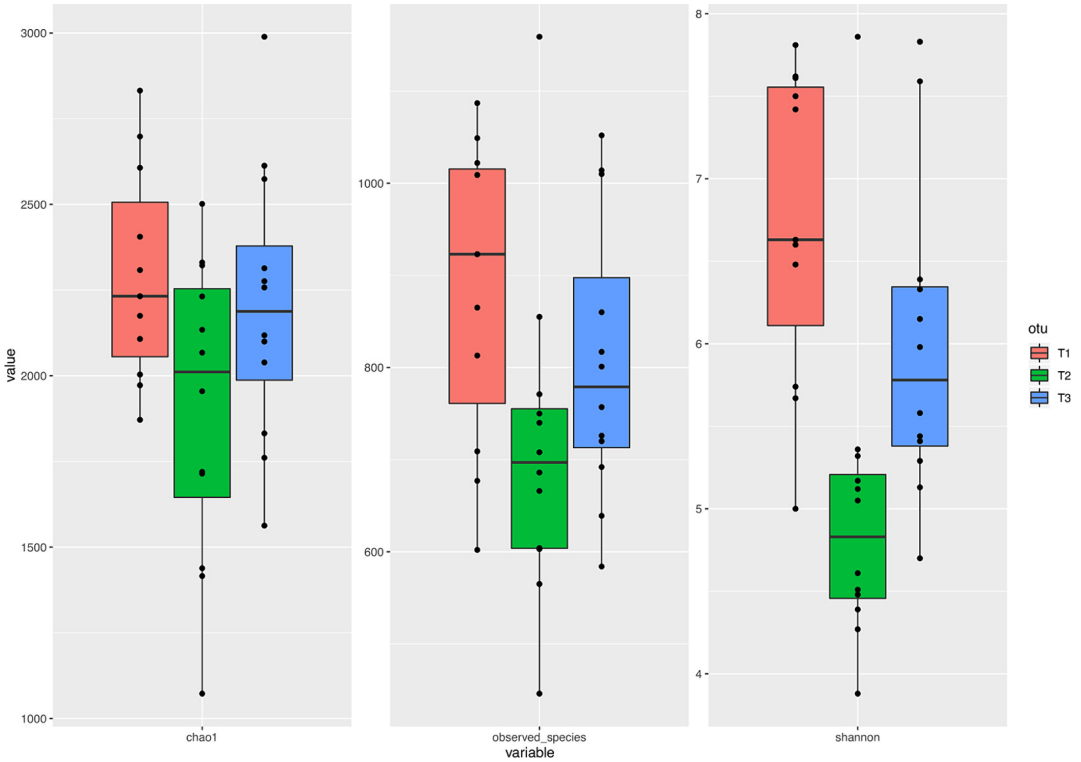

Fig. 5. Alpha diversity indices of the excreta microbiota of the laying hens depending on the sampling time (T1 = day 0; T2 = day 45; T3 = day 90).

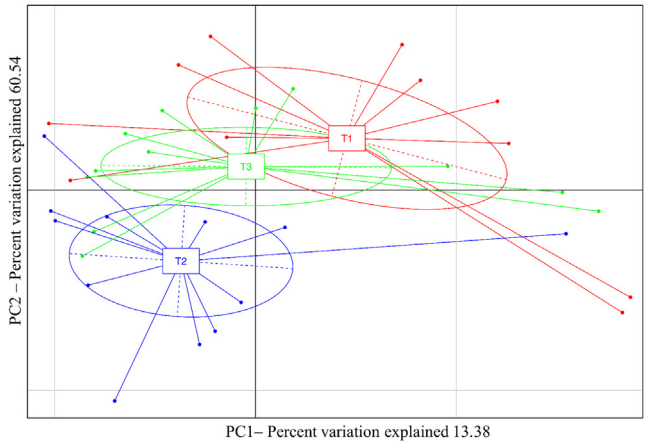

Fig. 6. Weighted UniFrac beta diversity, PCoA plots of the excreta samples of the laying hens collected on T1 (day 0), T2 (day 45), and T3 (day 90). PCoA = Principal Coordinate Analysis.

gastrointestinal response, to the novel aspect of their environment. In particular, despite the relatively low intake of DM from the larva biomass, the volume of larvae ingested might have been expected to modify the intestinal transit time, and thus have an indirect, negative influence on the DFI. However, the administration of live BSF larvae actually had a positive influence on the BW of BS hens. Considering that BP hens are generally heavier than their BS equivalents (Soglia et al., 2020), we might speculate that a more pronounced effect of the BSF supplementation would be observed on a smaller bird, but additional data will be needed to confirm or refute this hypothesis. Interestingly, the LR was higher and the FCR lower in the BSF-fed BP hens with respect to the BSF-fed BS hens. This may reflect a greater predisposition of the BP breed to nutritionally utilise the insect larvae. However, the absence of differences between the C and the BSF groups, as already underlined by Star et al. (2020), is in line with the absence of any changes to egg production in both the breeds. Independently of administration of live BSF larvae, the EW progressively increased over the course of the experimental trial, as physiologically observed during the normal production cycle (Travel et al., 2011). Furthermore, a higher EW was also identified in the BP hens compared with BS hens, probably reflecting the greater BW of the former (Soglia et al., 2020). Finally, the BP breed was also characterised by its lower DFI compared with the BS hens. Considering that brown-egg-laying strains typically show higher BW and feed consumption than white-egg-laying strains (NRC, 1994), this result appears contradictory. However, an autochthonous breed should not be compared to a highly-selected strain, further highlighting the need for additional studies.

The administration of live BSF larvae improved the plumage status of the BP hens only, as demonstrated by the higher (better) total integument scores identified in the BSF-fed hens compared with the C group. Although the differences in BP among treatments were statistically significant, the magnitude of these differences was relatively small. However, it is plausible that these differences could become more pronounced with an extended duration of larvae administration. This lies in agreement with the findings of Star et al. (2020), who also observed a reduction in plumage damage following the provision of live BSF larvae over a 12-week period. Previous studies revealed that providing laying hens with foraging materials or objects to peck at led to a reduction in severe feather pecking (as observed here) and, by consequence, an improvement in their plumage status (Huber-Eicher and Wechsler, 1998; Iqbal et al., 2020). Indeed, live insects constitute a natural substrate for the hens to peck at, meaning that the birds need not resort to pecking one another (Star et al., 2020). The fact that only the BP laying hens displayed an improvement in the integument scores may be related to their overall better plumage status in comparison with the BS breed, which probably made it easier to ameliorate a condition that was already good. Indeed, only the BS laying hens administered with live BSF larvae showed integument scores that were higher than that of C hens. Thus, we might need to consider a longer period of insect provision to observe a significant improvement in plumage that was of lower initial quality. Finally, the progressive worsening of the integument scores observed throughout the experimental trial was unexpected, as constant exposure to humans has previously been reported to reduce the level of fear in hens and, in turn, severe feather pecking and damage (de Haas et al., 2014). However, a total score ≥18–20 usually indicates good feather cover (Tauson et al., 2015), thus suggesting that the plumage status of the hens was already good.

Behavioural observations

The present study analysed video recordings executed 10 min prior to the provision of live larvae, making it possible to detect any possible anticipation effect, which must then be considered in the interpretation of the results. The laying hens of the current research displayed an increase in the frequency of grooming and allopreening behaviours following the administration of the live BSF larvae, as well as a reduction in severe feather pecking. Furthermore, the BSF-fed laying hens spent more time walking than the hens receiving the commercial diet only. According to Ipema et al. (2020), preening and allopreening can be classified as a "comfort behaviour", trotting, wing flapping and walking as "activity behaviours", and severe feather pecking as an "agonistic behaviour". Although severe feather pecking is not motivated by aggression (Savory, 1995), the receiver hen usually shows agonistic behaviour as a consequence of the pecking- and pulling-related pain experienced (Bilcík and Keeling, 2000). Based on these considerations, the use of BSF live larvae as environmental enrichment can promote the expression of positive behaviours (and thus increased bird comfort and activity) at the expense of negative ones (severe feather pecking towards conspecifics). This partially agrees with previous studies assessing the impact of live insects on poultry species since increased activity has been identified in both laying hens and broiler chickens fed BSF- or yellow mealworm-enriched diets (Pichova et al., 2016; Star et al., 2020; Ipema et al., 2020). Ipema et al. (2020) attributed this shift in the bird behavioural repertoire towards more active, natural behaviours to the stimulation of foraging activity. Furthermore, behaviours such as grooming and allopreening, performed when animals are not engaged in activity, can be used as direct evaluators of the birds' comfort status while waiting for larva administration (Li et al., 2020). This can also influence the expression of damaging behaviour such as severe feather pecking, which can be reduced as a consequence. Although the observations refer to the moment just prior to the consumption of larvae, meaningful considerations can be drawn and extended to the broader situation. If the provision of larvae is able to decrease severe feather pecking by virtue of its "distracting" effect, the staggered distribution of larvae throughout the day might also help to ameliorate this welfare issue in dual-purpose breeds characterised by high reactivity. However, the unaffected foraging behaviours (such as ground pecking and scratching) observed in the present study support the hypothesis put forward by Veldkamp and van Niekerk (2019), according to whom pecking at the larvae is, in itself, a rewarding type of explorative behaviour, which in turn reduces the need for ground pecking. The same authors also observed a reduction in aggressive pecking behaviour but no clear differences in terms of feather pecking, although feather and skin damage

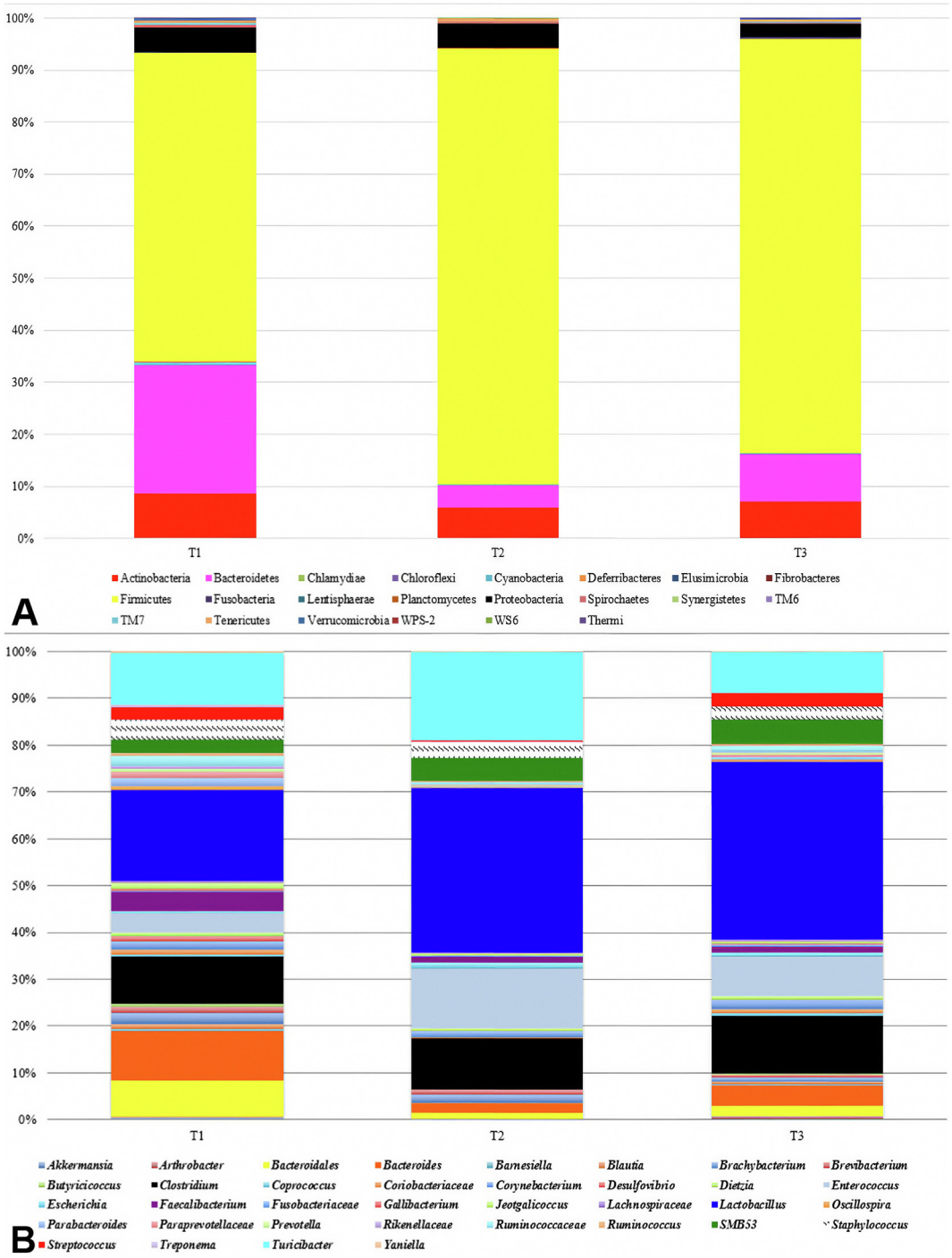

Fig. 7. Relative abundance of the main bacterial (**A**) phyla and (**B**) genera in the excreta samples of the laying hens collected on T1 (day 0), T2 (day 45), and T3 (day 90).

tended to be lower in the BSF-administered turkey poults studied (Veldkamp and van Niekerk, 2019). The remarkable decrease in severe feather pecking highlighted in the current research may be related to the bird genotype, as autochthonous breeds could respond to a novel pecking object or foraging material more effectively than highly selected strains (such as broilers or turkeys). Furthermore, the adoption of rearing conditions resembling those used in free-range farming (especially in terms of low stocking densities) may facilitate such a response. Another interesting aspect to underline is that the BS laying hens only displayed an increase in allopreening frequency after the administration of live BSF larvae. Considering that the BS breed is reported to be a more stressful and fearful breed than BP (De Marco et al., 2013), the stimulation of comfort behaviour though the creation of an enriched – and, therefore, more natural-like – rearing environment may act as an ideal social strategy for counteracting the effects of stressors. Independently of the administration of live BSF larvae, the high amount of time spent standing still at the expense of laying down observed throughout the experimental trial may have been related to the hot weather. As a final aspect to consider, the BP hens spent less time laying down than the BS hens, potentially underlying the presence of a breed-related difference in their beha-

vioural time budget, further research is necessary to deepen the understanding of this aspect.

Excreta corticosterone analysis

Despite showing the capacity for live BSF larvae to positively influence hen behaviour and plumage status, we did not reveal any differences between the groups in terms of ECM. The measurement of ECM is a well-recognised non-invasive, stress-free method for quantifying the effect of stress on daily corticoid rhythms (Carere et al., 2003) without interrupting the individual animal's behaviour (Hirschenhauser et al., 2012). However, many animal- and environment-related factors need to be taken into account to allow for the accurate interpretation of the results (Alm et al., 2014). As a partial confirmation of this aspect, both the breed and the time influenced the values of ECM recorded in the present study. In particular, the higher value of ECM obtained in the BS hens compared with the BP hens can reasonably be attributable to the above-mentioned more stressful nature of these hens, which are more easily frightened. Furthermore, the progressive increase in the ECM throughout the experimental trial could be related to the hot weather, as exposition to high environmental temperatures has previously been reported to activate the bird hypothalamic–pituitary–adrenal axis and, in turn, elevate corticosterone production (Lara and Rostagno, 2013).

Excreta microbiota characterisation

The administration of live BSF larvae did not lead to any changes in the excreta microbiota of the laying hens in the present study. This result is reasonable considering that the fresh insect larvae mainly contain water, and the concentration of nutrients was probably not sufficient to exert any of the effects on gut health previously associated with the use of BSF meal (Borrelli et al., 2017). However, independently of BSF administration, the excreta microbiota was distinctly influenced by both time and (to a lesser extent) the breed. In particular, on day 45, we identified a reduction in the bacterial richness and diversity – an important indicator of proper immunological and gut protective functions (Lu et al., 2003) – as well as specific changes in the microbial composition. At the phylum level, the relative abundances of Fusobacteria, Verrucomicrobia, and Bacteroidetes were all decreased, whereas Firmicutes displayed the opposite trend. High relative abundances of Fusobacteria have recently been highlighted in strains characterised by high egg-laying performance (Elokil et al., 2020), with some members of Verrucomicrobia also being recognised as performance-linked species in the chicken caecum (Torok et al., 2011). Furthermore, the relative abundances of *Lactobacillus*, *Streptococcus*, and *Turicibacter* amplicon sequence variants were increased at day 45, whereas Fusobacteriaceae and *Blautia* showed the opposite trend. *Lactobacillus* and *Streptococcus* are two well-known probiotic bacteria, whose characteristic functions include competitive exclusion, antagonism, bacterial interference, a barrier effect, modulation of the host immune system, and colonisation resistance (Khan and Chousalkar, 2020). Fusobacteriaceae and *Blautia* are another two amplicon sequence variants considered to exert beneficial effects on the health status of the gut, as the former activates host inflammatory responses to protect against pathogens that promote tumour growth (Kelly et al., 2018), and the latter is recognised as a butyrate-producer capable of alleviating gut inflammation (Wang et al., 2019). By contrast, *Turicibacter* has previously been reported to be negatively correlated with EW and LR in elderly laying hens (Gan et al., 2020). Therefore, the above-described changes are indicative of a partial, negative modulation of the excreta microbiota at the mid-point of the experimental trial. Similar to the results for the excreta corticosterone analysis, such alterations may be related to the hot weather, as environmental temperature is one of the main factors influencing the poultry gut microbiota (Sohail et al., 2015). However, these changes disappeared at the end of the experimental trial, thus potentially suggesting the birds' adaptation to the climatic conditions. Furthermore, independently of the sampling time, the excreta microbiota of the laying hens of the current research was mainly colonised by Firmicutes, Bacteroidetes, and Actinobacteria, as well as *Lactobacillus*, *Turicibacter*, *Bacteroides*, *Clostridium,* and *Enterococcus*, which are physiological in chickens (Khan et al., 2020). As a final aspect to highlight, the excreta microbiota of the BP hens showed an increase in the relative abundances of *Lactobacillus* and *Streptococcus* compared with BS hens, thus confirming the role of host factors (such as breed or strain) in affecting the chicken gut microbiota within any set environment (Kers et al., 2018).

Study limitations

Given the farming practices of these dual-purpose breeds, which are classified as endangered with a limited number of available individuals, it was feasible to establish only three replicates per experimental treatment per breed. However, it is important to underline that the main goals of the present study were to assess the welfare status of two native, dual-purpose breeds and to look for any differences in the responses to an environmental enrichment stimulus attributable to chicken genotype, rather than to assess their productive performances. The number of replicates may have partially influenced the statistical outcomes, thus making further research on this topic recommended. Furthermore, this study is the first on the use of live insect larvae as environmental enrichment in native, dual-purpose breeds, thus partially attenuating the above-mentioned limitations.

Conclusions

In conclusion, the administration of live BSF larvae as environmental enrichment positively influences the welfare of both BP and BS laying hens in terms of favouring the expression of positive behaviours and reducing severe feather pecking. We also highlight the potential for improved productive performance and plumage status in the BP breed. The identification of positive outcomes in relation to the low supplementation level herein adopted (6%), the birds' genetics and predisposition to free-range conditions are compatible with the concrete possibility of valorising these niche productions with the use of insects. On the other hand, the differences revealed between the two breeds underscore the importance of conducting further research with other native chicken breeds to determine whether the observed patterns hold true and to confirm the general outcomes.

Ethics approval

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, were adhered to and that appropriate ethical review committee approval was received. Specifically, the experimental protocol (Prot. No. 814715) was approved by the Bioethical Committee of the University of Turin (Italy). The authors confirm to have followed EU standards for the protection of animals used for scientific purposes.

Data and model availability statement

Sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (accession

number: PRJNA799198). Information can be made available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

None.

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Declaration of interest

None.

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