Poultry Science

Live black soldier fly (*Hermetia illucens*) larvae in feed for laying hens: effects on hen gut microbiota and behavior

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ABSTRACT This study examined the effects of including live black soldier fly (**BSF**, *Hermetia illucens*) larvae in the diet of laying hens on gut microbiota, and the association between microbiota and fearfulness. A total of 40 Bovans White laying hens were individually housed and fed 1 of 4 dietary treatments that provided 0, 10, 20%, or ad libitum daily dietary portions of live BSF larvae for 12 wk. Cecum microbiota was collected at the end of the experiment and sequenced. Behavioral fear responses to novel objects and open field tests on the same hens were compared against results from gut microbiota analyses. The results showed that the bacteria genera *Enterococcus, Parabacteroides*, and *Ruminococcus torques* group were positively associated with increased dietary portion of live larvae, while *Lactobacillus, Faecalibacterium, Bifidobacterium, Subdoligranulum*, and *Butyricicoccus* were negatively associated with larvae in the diet. Inclusion of larvae did not affect fear behavior, but the relative abundance of *Lachnospiraceae CHKCI001* and *Erysipelatoclostridium* was associated with fear-related behaviors. Further studies are needed to determine whether the change in gut microbiota affects fearfulness in the long−term.

Key words: live black soldier fly larvae, microbiota, behavior, laying hen

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INTRODUCTION

There is growing interest in using insects as a source of protein in poultry feed. Black soldier fly (**BSF**, *Hermetia illucens*) larvae are capable of converting substantial amounts of organic matter, such as food waste or animal manure, into edible proteins and fats during growth (Raksasat et al., 2020). Use of various organic waste sources for rearing can yield larvae with high-quality protein content ranging from 41 to ∼54% (Bava et al., 2019). Therefore, BSF larvae can be a viable alternative to conventional protein sources in feed for poultry (Abd El-Hack, 2020; Lu et al., 2022), with the potential to replace ingredients such as soybean meal and fish meal (Makkar et al., 2014; Tahamtani et al., 2021).

Multiple studies have shown that inclusion of BSF larvae (live, full-fat, or partially or completely defatted) in poultry diets benefits poultry growth, performance, nutrient digestibility, resistance to pathogens, and gut microbiota (Józefiak et al., 2018; Moula et al., 2018; De Souza Vilela et al., 2021; Ndotono et al., 2022a). This may be attributable to natural antibiotic properties of BSF larvae, which contain substances with antimicrobial activity such as lauric acid and chitin, altering the microbial community in the gut by reducing harmful bacteria such as *E. coli* and *Salmonella*, thereby promoting poultry health (Erickson et al., 2004; Lee et al., 2018). Dabbou et al. (2021) also suggested that modified BSF larvae fat showed a positive modulation of fecal microbiota by reducing potentially pathogenic bacteria such as *Clostridium* and *Corynebacterium*, without affecting intestinal morphology and performance of broiler chickens. Providing broilers with live insect larvae corresponding to 5% of the expected daily feed intake can slightly improve cecal microbiota by enhancing a minor fraction of short chain fatty acid-producing taxa (Colombino et al., 2021). Furthermore, providing live larvae as an environmental enrichment may also benefit poultry welfare, as previously observed in broilers, where live larvae reduced frustration in birds by stimulating foraging behavior and increasing activity levels (Biasato et al., 2022),while also decreasing the

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time spend in tonic immobility, indicating reduced fearfulness (Ipema et al., 2020). Understanding gut microbiota changes due to factors such as diet is crucial to bird health. The gut microbiota of laying hens plays an important role in immunity and nutritional metabolism, and may also affect fear-related behaviors (Polansky et al., 2016; Yan et al., 2021). For instance, a study on Japanese quail found that transfer of gut microbiota from birds of a line with low emotional reactivity to birds of a line with high emotional reactivity resulted in reduced fear responses in tonic immobility and novel environment tests early in life (15 d of age), but increased fear responses 2 wk later (Kraimi et al., 2019). Probiotic administration of *Bacillus amyloliquefaciens* has been shown to reduce distress calls and aggressive behaviors in turkeys (Naglaa, 2013), while addition of probiotics (*Enterococcus faecium* and *Lactobacillus reuteri*) to the diet can reduce fear response in broilers under heat stress (Mohammed et al., 2021). It is worth mentioning that excessive fear is associated with increased stress sensitivity, decreased performance, weight loss, and reduced feed intake in laying hens (Uitdehaag et al., 2008; De Haas et al., 2013).

The starting hypothesis in the present study was that provision of live BSF larvae to laying hens would modulate the gut microbiota. To test this, gut microbiota from individual hens fed different levels of BSF larvae were deep-sequenced, to evaluate effects on the gut microbiome. The data obtained were compared with results from fear tests performed on the same individuals, previously reported in Tahamtani et al. (2021), to search for possible correlations between gut microbiota and fearfulness.

MATERIALS AND METHODS

Full details of the experimental set-up and fearfulness tests can be found in Tahamtani et al. (2021). A brief description is provided below.

Birds, Housing, and Diets

A total of 40 Bovans White pullets were acquired from a commercial breeding farm (Närkesberg Hönseri AB, Åsbro, Sweden) at 16 wk of age. The hens were individually housed in raised pens (150 cm × 75 cm, L × W) at the Swedish Livestock Research Center, Lövsta. The pens had a solid floor and were equipped with a nest box, a perch, wood shavings, separate feeding troughs, and nipple drinkers.

At 19 wk of age, the hens were randomly divided into 4 dietary treatments ($n = 10$ per group): Control—standard concentrate feed; L10, L20—a daily portion of live BSF larvae providing 10 or 20% of expected daily dry matter (**DM**) intake (Bovans 2020) and a complementary pelleted concentrate; and Ad Lib—ad libitum access to live BSF larvae, complementary pelleted concentrate, and soy mash. The pelleted concentrates, live larvae, and soy mash (only in the Ad Lib diet) were provided in separate bowls/feed troughs. In addition, all hens had ad libitum access to grit. The composition of the control feed and of the complementary pelleted concentrate used in treatments L10, L20 and Ad Lib is shown in Table S1 in Supplementary Material to Tahamtani et al. (2021). The larvae used in the present experiment were produced and portioned as described in Tahamtani et al. (2021).

Open Field Test

An open field (**OF**) test was conducted at 29 wk of age. In brief, the behavioral responses of the birds were evaluated in a novel open field located in a room adjacent to the home room, to prevent them from hearing or seeing other birds. The field consisted of a 1 m × 1 m arena with 60 cm of solid walls and 70 cm of wire mesh walls above the solid walls. The top was partly covered with wire mesh to prevent birds from escaping, while still providing a clear image of the arena for a video camera installed above. Individual birds were transported to the test room in the arms of the experimenter and placed in the middle of the arena, with the lights off to prevent them from escaping. After the experimenter left and the lights were turned on, the hen was video recorded for 10 min. The performance of the hen in terms of pacing, gakel calls, total number of transitions between the inner and outer zones of the arena, and number of fecal droppings were counted in the video recordings, by an observer blind to the treatment. Lower frequencies of pacing, gakel calls, and fecal droppings were interpreted as signs of less fearfulness in the hens (Jones and Waddington, 1992; Hocking et al., 2001).

Novel Object Test

A novel object (**NO**) test was performed at 30 wk of age. Briefly, the behavioral response of the birds to a NO was video recorded for 10 min. Two NOs (a colored wooden stick and an orange bottle) were used in the test, to prevent habituation of hens in adjacent cages. The NO was placed in front of the nest box and observations began 30 s after the start of video recording. An observer, blind to the treatments, scored time spent in the half of the cage closest to the NO. Longer time spent close to the NO was interpreted as a sign of less fearfulness in the hens (Jones, 1987; Forkman et al., 2007).

Gut Sample Collection

At 31 wk of age, all hens were killed by intravenous administration of pentobarbital (Allfatal vet. 100 mg/mL, Omnidea AB, Stockholm). Cecum contents were carefully collected immediately under aseptic condition and placed in liquid nitrogen before storage at −80°C for later analysis.

DNA Extraction and DNA Qualification and Bioinformatic Analysis

The digesta samples were thawed on ice and used for DNA extraction with a PowerFecal Pro DNA Kit (Qiagen, Germany). In brief, 180 mg cecum contents and 800 μL solution CD1 (provided with the kit) were added to a PowerBead Pro tube (provided with kit), followed by beating on a Precellys evolution homogenizer (Bertin Technologies SAS, France) at 8,000 rpm for 2 × 60 s with a 30 s pause. The PowerBead Pro tube was then centrifuged at 15,000 × g for 5 min, and the supernatant was retrieved and treated following the manufacturer's protocol. DNA was eluted with 60 μL elution buffer C6 (provided with kit) and stored at −20°C for delivery to Novogen (Cambridge, UK). The sequencing library of the 16S rRNA gene was constructed and sequenced at Novogen, using the illumina NovaSeq platform. The V4 region of the 16S rRNA gene was amplified with primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT). The raw sequencing data have been deposited in the Sequence Read Archive at the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/sra), under accession number PRJNA961026. Processing of the 16S rRNA gene sequencing data was performed as described elsewhere (Sun et al., 2022), with the following modifications: 1) truncation length of 220 bp for both forward and reverse reads; 2) the amplicon sequence variants (**ASV**) table was rarefied at 46,786 reads per sample; 3) the SILVA SSU Ref NR 99 138 dataset was used for taxonomic classification (Quast et al., 2013); and 4) the generalized UniFrac distance matrix (alpha = 0.5) was generated using the QIIME2 diversity plugin (Bolyen et al., 2019).

Ethical Statement

All experimental procedures involving animals were approved by the ethics committee for the Uppsala region of the Swedish Board of Agriculture (application number 5.8.18-03402/2020).

Statistical Analysis

The rarefied ASV table was used to calculate the number of observed ASVs, Faith's phylogenetic diversity, Simpson and Shannon index. The Kruskal-Wallis rank test, followed by Dunn's test for pairwise comparisons with Benjamini & Hochberg (**B−H**) correction, was used to check for statistically significant differences in observed ASVs, Faith's phylogenetic diversity, Simpson and Shannon index between treatment groups, using the QIIME2 q2-diversity plugin (Kruskal and Wallis, 1952; Dunn, 1964; Benjamini and Hochberg, 1995). Permutational multivariate analysis of variance (**PERMANOVA**) of generalized UniFrac distance matrix with B-H correction was conducted to evaluate differences between the dietary treatment groups, using the q2-diversity plugin (Anderson, 2001). To investigate the effect of dietary treatment on gut microbiota, Quasi−Poisson generalized linear model was used for significance analyses and Tukey HSD was used for multiple pairwise comparisons, using R software (Tukey, 1977; Ver Hoef and Boveng, 2007). Spearman correlation analysis was conducted to investigate markers of interest in cecal microbiota (the top 14 relative abundance genus) and behavioral data on laying hens. In the first step, the microbiota data and behaviors of all birds were included in the analysis. In the second step, the correlation analysis was conducted within each dietary treatment group, to avoid any potential interference caused by larval feed. R software was used to graph the data (R Core Team, 2021).

RESULTS

The sequences obtained in analysis of microbial ASVs from cecum samples were distributed in 695 ASVs, representing 57 taxonomical families and 145 genera. The rarefaction curve generated from the number of observed ASVs indicated sufficient sequencing depth, with the Ad Lib treatment group exhibiting the highest level of observed ASVs (Kruskal-Wallis, $P < 0.01$) (Figure 1A). With increasing provision of larvae in the diet (L10, L20, Ad Lib), Shannon index for gut microbiota in birds gradually increased compared with the Control birds. The Ad Lib and Control groups exhibited the highest and lowest Shannon index value, respectively ($P < 0.001$) (Figure 1C). Similarly, the Faith's phylogenetic diversity revealed the highest species richness was observed in Ad Lib group ($P < 0.01$) (Figure 1D). The Simpson index was significantly higher in L10 and L20 groups compared to control ($P < 0.05$), yet the absolute difference was very small (Figure 1E). As revealed by these alpha diversity indices, provision of live BSF larvae increased the alpha diversity of species in the gut.

Beta diversity, as revealed by the generalized UniFrac distance-based principal coordinate analysis (**PCoA**) plot, revealed an effect of dietary treatment (Figure 1B). Differences in microbiota composition mainly appeared in PC1 with increased proportion of BSF larvae in the diet. PERMANOVA and post hoc analysis revealed significant differences between all possible pairwise comparisons in all groups ($P < 0.05$).

Population analysis revealed that the same top 10 genera were present in all 4 treatment groups, but that their relative abundance differed (Figure 2). The relative abundance of the top 10 genera in the Control, L10, L20, and Ab Lib groups was as follows: *Lactobacillus* 12.94 to 28.14%, unclassified *Lachnospiraceae* 15.91 to 18.13%, *Faecalibacterium* 5.67 to 9.89%, *Ruminococcus torques* group 4.08 to 6.35%, *Parabacteroides* 3.06 to 8.55%, *Bacteroides* 2.80 to 4.60%, *Bifidobacterium* 1.17 to 4.28%, *Blautia* 2.05 to 2.45%, *Alistipes* 2.03 to 2.38%, and *Oscillospiraceae-UCG-005* (1.38−3.34%).

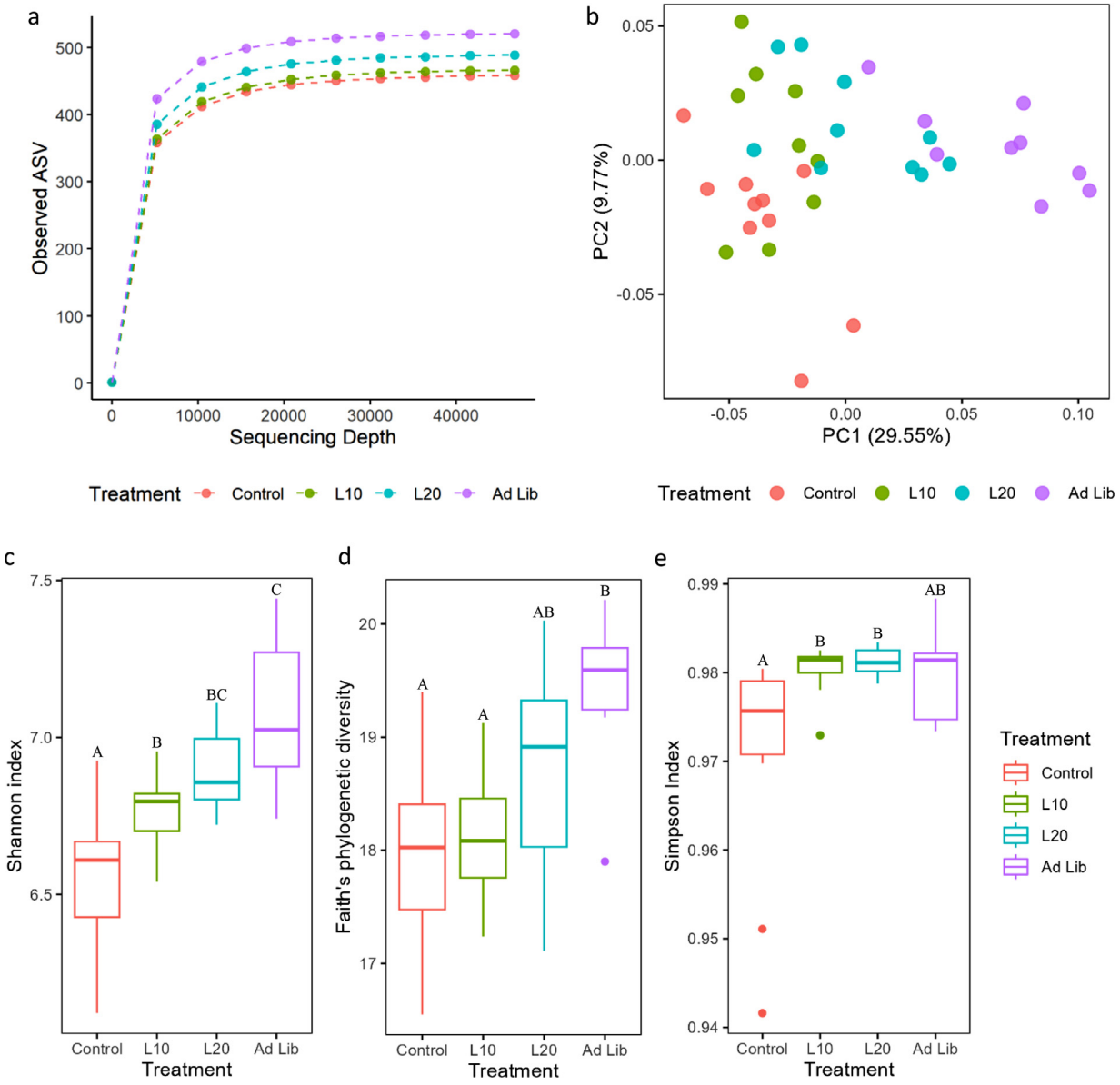

Figure 1. (A) Rarefaction curves of observed amplicon sequence variants (ASV) in cecal contents of laying hens fed different diets containing 0% (Control, standard concentrate), 10% (L10), 20% (L20), and ad libitum (Ad Lib) black soldier fly larvae; (B) principal coordinate analysis (PCoA) plot showing differences in generalized UniFrac distance matrix for the different dietary treatments; (C–E) boxplot of Shannon, Faith's phylogenetic diversity and Simpson index for the different dietary treatments; different superscript letters $^{A-C}$ indicated significant differences between dietary treatments ($P < 0.05$).

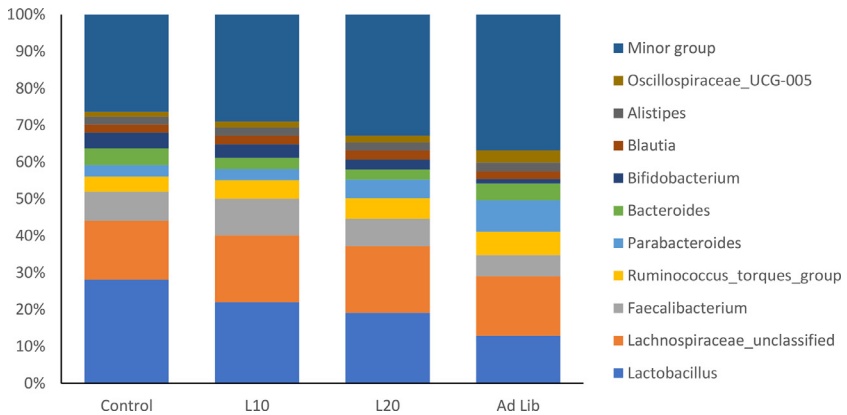

Figure 2. Relative abundance (%) of the top 10 bacteria genera present in cecal contents of laying hens fed different diets containing 0% (Control, standard concentrate), 10% (L10), 20% (L20), and ad libitum (Ad Lib) black soldier fly larvae.

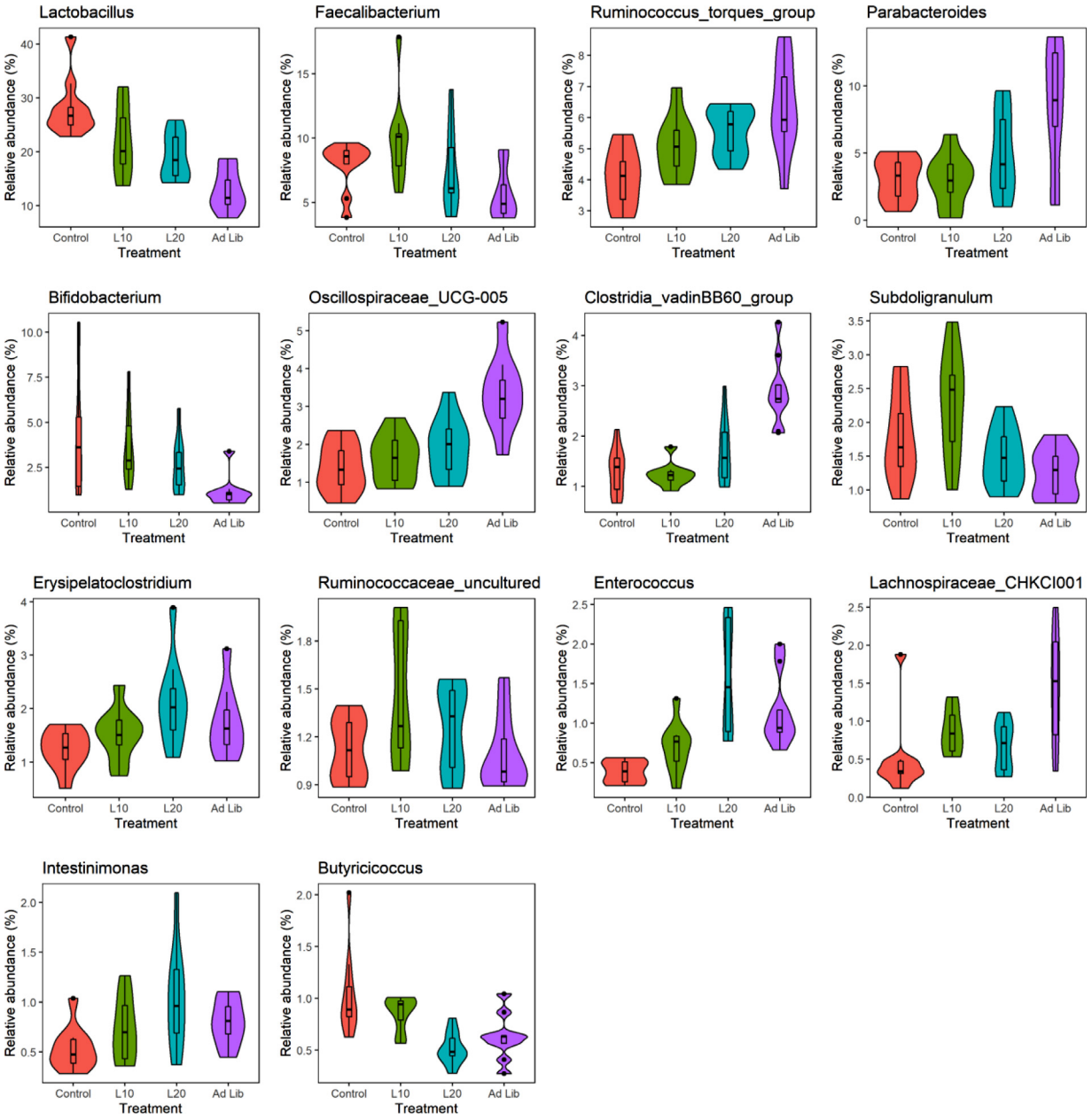

Figure 3. Violin plots (including box with median and interquartile range), of relative abundance (%) of the top 14 genera that differed significantly in cecal contents of laying hens fed different diets containing 0% (Control, standard concentrate), 10% (L10), 20% (L20), and ad libitum (Ad Lib) black soldier fly larvae. Violin width corresponds to the distribution of samples.

To investigate bacterial groups potentially responsible for the differences in gut microbiota diversity between the groups, a Quasi−Poisson generalized linear model was used to identify differences in relative abundance of the top 14 bacterial genera altered by dietary larvae treatment (Figure 3, Table 1). With increasing provision of larvae, the relative abundance of *Lactobacillus* and *Bifidobacterium* in the gut of birds gradually decreased compared with the Control birds, while the abundance of *Ruminococcus torques* group, *Oscillospiraceae UCG-005, Clostridia vadinBB60* group, and *Parabacteroides* gradually increased. However, compared with the Control group, the L10 group only had significantly increased abundance of uncultured *Ruminococcaceae*, while the L20 group had significantly increased abundance of *Erysipelatoclostridium, Enterococcus*, and *Intestinimonas*, and reduced abundance of *Butyricicoccus*. Ab libitum access to BSL larvae significantly affected the abundance of all top 14 genera except for *Faecalibacterium, Subdoligranulum, Erysipelatoclostridium*, uncultured *Ruminococcaceae*, and *Intestinimonas* (Table 1). Within the 3 dietary larvae treatments, the Ad Lib group had the lowest abundance of *Lactobacillus* and the highest abundance of *Parabacteroides, Oscillospiraceae UCG−005*, and *Clostridia-vadinBB60* group. The L20 and Ad Lib groups had the lowest abundance of *Bifidobacterium* and *Butyricicoccus*, and the highest abundance of *Enterococcus* (Table 1).

To visualize possible links between the behavior of individual hens and the gut microbiota, we conducted Spearman correlation analysis of differential bacteria based on degree of contribution of the top 14 genera and

Table 1. Estimated marginal mean (±SE) of sequencing counts of the top 14 genera[1] that differed in cecal contents of laying hens fed different diets containing 0% (Control, standard concentrate), 10% (L10), 20% (L20), and ad libitum (Ad Lib) black soldier fly larvae.

Genera	Control	L10	L20	Ad Lib
Lactobacillus	[2]9.49 ± 0.07[c3]	9.24 ± 0.08[bc]	9.10 ± 0.08[b]	8.71 ± 0.11[a]
Faecalibacterium	8.22 ± 0.11[ab]	8.44 ± 0.10[b]	8.15 ± 0.11[ab]	7.88 ± 0.14[a]
*Ruminococcus_torques_*group	7.55 ± 0.07[a]	7.77 ± 0.07[ab]	7.86 ± 0.06[b]	8.00 ± 0.06[b]
Parabacteroides	7.28 ± 0.23[a]	7.27 ± 0.25[a]	7.76 ± 0.18[ab]	8.29 ± 0.15[b]
Bifidobacterium	7.60 ± 0.18[b]	7.44 ± 0.20[b]	7.12 ± 0.23[ab]	6.31 ± 0.36[a]
Oscillospiraceae_UCG-005	6.47 ± 0.14[a]	6.64 ± 0.14[a]	6.80 ± 0.12[a]	7.35 ± 0.10[b]
*Clostridia_vadinBB60_*group	6.41 ± 0.11[a]	6.36 ± 0.12[a]	6.68 ± 0.10[a]	7.21 ± 0.08[b]
Subdoligranulum	6.72 ± 0.10[ab]	6.97 ± 0.09[b]	6.54 ± 0.11[a]	6.36 ± 0.13[a]
Erysipelatoclostridium	6.37 ± 0.13[a]	6.57 ± 0.12[ab]	6.90 ± 0.10[b]	6.70 ± 0.11[ab]
*Ruminococcaceae_*uncultured	6.26 ± 0.08[a]	6.53 ± 0.07[b]	6.38 ± 0.07[ab]	6.24 ± 0.08[a]
Enterococcus	5.20 ± 0.22[a]	5.78 ± 0.17[ab]	6.60 ± 0.11[c]	6.28 ± 0.13[bc]
Lachnospiraceae_CHKCI001	5.44 ± 0.24[a]	6.01 ± 0.19[ab]	5.74 ± 0.21[a]	6.51 ± 0.15[b]
Intestinimonas	5.51 ± 0.16[a]	5.83 ± 0.15[ab]	6.18 ± 0.12[b]	5.92 ± 0.14[ab]
Butyricicoccus	6.18 ± 0.09[c]	6.00 ± 0.10[bc]	5.48 ± 0.13[a]	5.68 ± 0.12[ab]

[1]The order of genera is arranged from high to low abundance.
[2]Values are estimated marginal mean ± standard error.
[3]Values within rows with different superscripts are significantly different ($P < 0.05$).

fear behavior observed in NO and OF tests. As reported previously in Tahamtani et al. (2021), the behaviors observed were not affected by dietary treatment. Thus, in the first step, in the present analyses of correlations between birds' behaviors and microbiota, all birds regardless of the dietary treatment group were included in the analysis. The results showed that pacing time was positively associated with *Lachnospiraceae CHKCI001* and that number of gakel calls had a positive correlation with relative abundance of *Lachnospiraceae CHKCI001, Clostridia vadinBB60* group, and *Erysipelatoclostridium*, and a negative correlation with *Lactobacillus* (Figure 4). In the second step, to avoid any potential interference caused by larval feed on the gut microbiota of the laying hens, the correlation analysis was conducted within each dietary treatment group. The result revealed that pacing time was positively associated with *Lachnospiraceae CHKCI001* in Control group (Figure S1a) and *Erysipelatoclostridium* in L20 group (Figure S1c). The number of gakel calls had a positive correlation with *Lachnospiraceae CHKCI001* in Ad Lib group (Figure S1d) and *Erysipelatoclostridium* in L20 group (Figure S1c). On the other hand, *Lactobacillus* and *Clostridia vadinBB60* group had no correlation with fear behavior (Figure S1).

DISCUSSION

This study demonstrated that feeding live BSF larvae to laying hens increased both alpha diversity, indicated by number of observed ASVs and Shannon index, and beta diversity, indicated by generalized Unifrac distance analysis. Similarly, previous studies investigating the effect of larvae-based diets have found that feeding BSF larvae-based diets does not have a negative effect on nutrient utilization and gut microbiota composition in poultry, and that diets containing higher concentrations of BSF result in higher microbial richness and diversity (Moula et al., 2018; Ndotono et al., 2022a). In our study, the dominant core microbiota in the cecum of laying hens belonged to *Lactobacillus*, unclassified *Lachnospiraceae, Faecalibacterium, Ruminococcus torques* group, *Parabacteroides, Bacteroides*, and *Bifidobacterium*, which accounted for 68.05% of the total in the Control group, 64.79% in the L10 group, 60.65% in the L20 group, and 55.42% in the Ab Lib group. This decrease in cecal core microbiota content was associated with increased microbial alpha diversity, suggesting that laying hens fed live BSF larvae may have healthier gut microbial communities, since high bacterial diversity

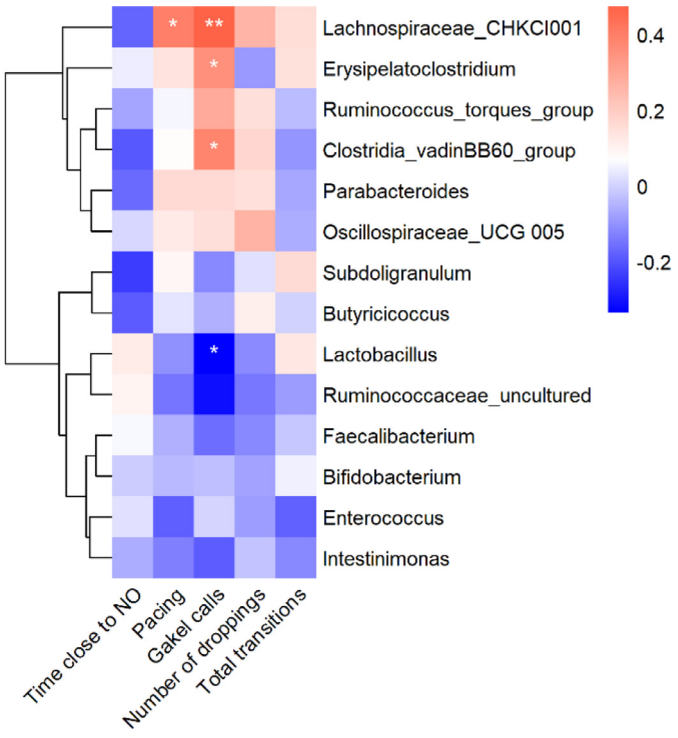

Figure 4. Spearman correlation coefficient for the relationship between fear-related behavior and different bacterial genera in the gut of laying hens. Heat maps show notable statistical correlation values (r), where red squares indicate significant positive correlation ($0 < r \leq 1$), white squares no correlation ($r = 0$), and blue squares significant negative correlation ($-1 \leq r < 0$). Deeper color hue indicates stronger correlation (*$P < 0.05$, ** $P < 0.01$).

tends to be associated with a healthy gastrointestinal tract (Borrelli et al., 2017). Higher bacterial diversity in the gut community may increase competition with pathogens for resources and colonization, preventing invasion and infection by pathogens, which can improve the overall health status of poultry (Yadav and Jha, 2019).

Regarding modulation of the gut microbiota, we observed a gradual increase in relative abundance of *Ruminococcus torques* group, *Parabacteroides, Oscillospiraceae UCG-005*, and *Clostridia vadinBB60* group with increased provision of live BSF larvae. These bacteria are commonly associated with immune regulation (Khan and Chousalkar, 2020; Liu et al., 2021; Dehau et al., 2023; Fan et al., 2023). For instance, increased mortality in poultry during subclinical natural occurrence of necrotic enteritis has been found to be accompanied by a decrease in abundance of *Ruminococcus torques* group (Emami et al., 2021). *Parabacteroides* is reported to promote anti-inflammatory regulatory T cell abundance by inhibiting the TLR4-Akt signaling pathway (Koh et al., 2018). In addition, several types of intestinal bacteria, such as *Lachnospiraceae CHKCI00, Intestinimonas, Faecalibacterium*, and *Butyricicoccus*, have been shown to produce short-chain fatty acids (**SCFAs**) (Rychlik, 2020; Zhou et al., 2022). SCFAs are known to play an important role in poultry gut health by inhibiting acid-sensitive pathogens and thus reducing the abundance of undesirable and harmful bacteria (Borrelli et al., 2017). These bacterial changes may be related to the larval component chitin, a naturally occurring polysaccharide that can be fermented by microorganisms, degraded by SCFA-producing bacteria, and used as substrate by other microorganisms (Luparelli et al., 2022). In this study, the abundance of bacteria *Lactobacillus* was reduced in the cecum of laying hens fed live BSF larvae, especially in the Ab Lib group. Similarly Józefiak et al. (2018) found that even small amounts (0.05−0.2%) of BSF larvae meal can reduce the abundance of *Lactobacillus* in broiler chickens. It is important to note, however, that live BSF larvae may act as a mechanical vehicle in transmission of beneficial or harmful strains of bacteria to poultry, resulting in colonization of the gut by these beneficial or harmful bacteria (Ndotono et al., 2022a). In the present study, the L20 and Ad Lib groups had the highest abundance of *Enterococcus* in the gut. This agrees with findings by Ndotono et al. (2022b) of higher abundance of the genus *Enterococcus* in the gut of broiler chickens fed diets containing BSF larvae compared with a control diet and highest abundance for a diet with 50% BSF larvae inclusion. Different roles of *Enterococcus* strains in the gut of poultry have been reported, for example, a study on broilers found that *Enterococcus faecalis* M74 strains significantly increased antioxidant status, serum calcium levels, and body weight in broilers (Capcarova et al., 2010). Another study found that *Enterococcus faecalis* ST100 is potentially virulent in poultry hosts, and this sequence type was recently identified as the cause of vertebral osteomyelitis lesions in poultry (Braga et al., 2018). However, our sequencing result was not sufficient to identify strains of *Enterococcus* with sequences below genus level, and further studies are needed to determine whether enrichment of *Enterococcus* in the gut of laying hens fed live BSF larvae has a positive or negative effect on hen health.

In the present study, it was not possible to separate the effects of administration of larvae as environment enrichment and changes in gut microbiota on the bird's behaviors. However, to minimize possible effects from environmental enrichment, the larvae were provided in a bowl with a brim that prevented escaping Tahamtani et al. (2021), and also made them easy for hens to pick. There was no observable impact of the dietary larvae treatments on the fearfulness behavior of laying hens as described by Tahamtani et al. (2021). However, some potential correlations between certain intestinal genera and hen behavior were observed. The OF test exposes hens to a new environment in social isolation, and in order to reinstate social contact the hens have to make calls and increase locomotion to search for conspecifics. Therefore less time spent pacing and fewer gakel calls are associated with higher fearfulness (Suarez and Gallup, 1983). Focusing on emotional state, we observed that lower expression of fearfulness (more gakel calls) was linked to decreased levels of *Lactobacillus* by dietary larvae treatment. This is similar to previous findings that *Lactobacillus* levels are lower in low-fear red junglefowl compared with high-fear junglefowl (Puetz et al., 2021). However, there is also some evidence that *Lactobacillus* can reduce fear response and fear-related behaviors (Bravo et al., 2011; Zakari et al., 2019). In addition, the correlation analysis within each dietary treatment revealed no association between *Lactobacillus* and fear behavior suggesting that the link between this genus and fearfulness needs to be further explored. In contrast to the observed association of *Lactobacillus* with fear behavior, we found that *Lachnospiraceae CHKCI001* and *Erysipelatoclostridium* were negatively associated with fearfulness in the hens. Interestingly, Vicentini et al. (2022) reported that anxiety-like and despair behaviors were positively associated with the family *Lachnospiraceae* in mice, while Huovinen et al. (2023) reported that fearfulness had negative associations with the genus *Erysipelatoclostridium* in infant girls. However, it is worth noting that our experiment only covered a 12−wk period and did not include the early life (rearing) stage, which is known to be crucial for behavioral development of the laying hen. It is possible that provision of live larvae and associated alterations in gut microbiota in early life would lead to stronger effects on the fear behavior of adult hens.

CONCLUSIONS

This study investigated the gut microbiota of laying hens fed live BSF larvae as part of their diet. Some variation in gut microbiota between individual hens within dietary groups was observed, but the dominant effect on

gut microbiota came from dietary treatment. Inclusion of live BSF larvae in the diet enriched intestinal microbial diversity and promoted growth of *Enterococcus, Parabacteroides*, and *Ruminococcus torques* group. On the other hand, some known beneficial bacteria, such as *Lactobacillus, Bifidobacterium*, and *Butyricicoccus*, were negatively associated with larvae in the diet. Correlation analysis showed that relative abundance of *Lachnospiraceae CHKCI001*, and *Erysipelatoclostridium* was related to fearfulness in the laying hens. Further investigation is needed to determine the long−term impact of including live BSF larvae in the diet of laying hens on gut microbiota and behavior.

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DISCLOSURES

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIALS

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