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RESEARCH ARTICLE

The effect of different levels of *Hermetia illucens* oil inclusion on caecal microbiota of Japanese quails (*Coturnix japonica*, Gould, 1837)

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Abstract

In this study, we investigated the effect of the dietary inclusion of *Hermetia illucens* larvae oil on the diversity and structure of the bacterial community of the caecal content of Japanese quails (*Coturnix japonica*). A total of 40 quails, equally selected for slaughter from 100 animals which were divided evenly into four treatment groups including control group (C) with a diet containing corn oil and 3 experimental groups with partial (25%, 50%) or total (100%) substitution of corn oil by *H. illucens* larvae oil, here referred to as Black soldier fly larvae oil (BSFO): BSFO 25, BSFO 50 and BSFO 100, respectively. After slaughtering (42 days of age), the microbiota of caecal samples was examined by high-throughput sequencing using the V4-V5 region of the 16S rRNA gene. In all the studied groups the dominant phylum was *Firmicutes* with prevailing families of *Ruminococcaceae* and *Lachnospiraceae*. Caecal microbiota was meaningly influenced on genus level. The linear discriminant analysis effect size (LefSe) analysis for the differential taxa abundance showed that *Lactobacillus* was significantly increased in BSFO 25 group, *Fusicatenibacter* was significantly enriched in all the experimental groups fed larvae oil (BSFO 25, 50 and 100) and *Subdoligranulum* was highly elevated in BSFO 100 group. The analysis revealed statistical dissimilarities between the control group (C) and the groups with 50% and 100% oil replacement (BSFO 50 and 100). The bacterial diversity was significantly suppressed in the samples of quails fed the diet with a total inclusion of *H. illucens* oil (BSFO 100). The results showed the considerable effect of Black soldier fly larvae oil on the caecal microbiota of Japanese quails.

Keywords

black soldier fly larvae oil – quails – caecal microbiota

1 Introduction

Poultry is nowadays the leading source of meat produced due to the remarkable feed conversion ratio, short lifecycle, and low greenhouse gas emission (FAO, 2020). Despite that chicken still dominate the poultry egg and meat production sectors, quails' production is a widely emerging branch in the poultry industry as it introduces diversity among both egg and meat yield and has been used extensively for both purposes (Minvielle, 2004; Nasr *et al.*, 2017; Sabow, 2020). In Asia, Japanese quails are typically reared for their egg yield, while in the US

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and Europe their meat production is the core driver and in some Countries, such as Turkey for instance, Japanese quails are produced for both eggs and meat (Narinc *et al.*, 2015). Reasons for interest in breeding these birds insist in their small size, rapid growth, early sexual maturity (7-8 weeks), short generation interval, and high laying rate (Du *et al.*, 2020; Huss *et al.*, 2008). In addition, quail are increasingly becoming an alternative to chicken, especially for health-conscious consumers, due to the higher levels of vitamins (A, C), minerals and amino-acids and lower content of fat and cholesterol in their meat (Fakolade, 2015; Glick and Fischer, 2013). Japanese quail (*Coturnix japonica*) moreover represents favoured animal model in the field of poultry research (Huss *et al.*, 2008) and due to the well-researched and confirmed genetic similarities with chicken (*Gallus gallus domesticus*) it has been adopted as the best optimised model to study the poultry functional genomics (Minvielle, 2009; Shin, 2017). However, the number of intestinal microbiota studies, which has been an important topic in recent years, is surprisingly limited in these animals.It is now well accepted that the microbiota of digestive tract contributes to the overall health status of animals and their productivity. Even if the primary role of the gastrointestinal tract (GIT) microbes is digestion of food substrates, the microbiota is important in many other functions ranging from defense against pathogens, production of nutrients to maturation and regulation of the immune system (Aruwa *et al.*, 2021; Carrasco *et al.*, 2019; Clavijo and Flórez, 2018).

Up to now only few studies on the characterisation of quail intestinal microbiota have been done (Du *et al.*, 2020; Ma *et al.*, 2021; Su *et al.*, 2014; Wilkinson *et al.*, 2016). They differ substantially in intent and goals, either characterising the bacterial community profile along the whole GIT (Du *et al.*, 2020; Su *et al.*, 2014; Wilkinson *et al.*, 2016), or focusing, from different reasons, on ileal microbiota (Borda-Molina *et al.*, 2020; Vollmar *et al.*, 2020), or describing composition of cultivable bacteria (Du *et al.*, 2020; Su *et al.*, 2014; Wilkinson *et al.*, 2016), or studying caecal (Liu *et al.*, 2015) or duodenal and ileal microbiota of atherosclerosis susceptible and resistant quail strains (Liu *et al.*, 2018). An interesting article of Ma *et al.* (2021) provided the description of the gene catalog of the caecal bacteria of Japanese quail including the comparison of bacterial taxa and predictive metagenomic functions of male and female quail (Ma *et al.*, 2021).

Research on altering the composition of the poultry intestinal environment with the use of insect feed additives as an alternative source of fats, proteins and/or antimicrobial peptides has made significant strides in recent years (Clavijo and Flórez, 2018; Shin, 2017). *Hermetia illucens* (also called black soldier fly) and *Tenebrio molitor* are the most studied insect species in poultry nutrition (Benzertiha *et al.*, 2020; Bovera *et al.*, 2018; Colombino *et al.*, 2021; Kierończyk *et al.*, 2018; Moniello *et al.*, 2019; Secci *et al.*, 2018, 2022) One of the major benefits of this insect is the ability to decompose organic waste to animal feed as a dietary source (Addeo *et al.*, 2021; Surendra *et al.*, 2016).

Corn production has previously showed negative environmental consequences, such as gas emissions, deforestation, and high-water costs (Holka and Bieńkowski, 2020), which is not the case for the environmentally friendly production of black soldier fly (BSF) feed materials, based on the use of plant-origin wastes, as a source of nutrients in vertical rearing systems, to limit greenhouse gas (GHG) emissions and to use frass as a fertiliser (Józefiak *et al.*, 2016). Despite the higher cost of BSFO compared to corn oil, it can be employed as the basis of a highly promising technology to sustain a circular economy (Barragan-Fonseca *et al.*, 2017). Up to date, only one study compared the usage of BSFO as an alternative source of corn oil in poultry, but the study focused on growth performance, serum parameters and carcass characteristics Kim *et al.*, 2020).

The fatty acid (FA) composition of Black soldier fly larvae (BSFL) predominantly comprises saturated fatty acids (SFAs), such as lauric and palmitic acids (Ewald *et al.*, 2020; Kim *et al.*, 2020; Makkar *et al.*, 2014; Zotte *et al.*, 2019). Dietary medium-chain fatty acids (MCFA), and in particular lauric acid, have a positive effect on poultry gut microbiota by having an antimicrobial effect, which can thus stimulate the health of the gastrointestinal tract by inhibiting potentially pathogenic bacteria (Baltic *et al.*, 2019; Boyen *et al.*, 2008; van der Hoeven-Hangoor *et al.*, 2013; van Immerseel *et al.*, 2006). The lauric acid has shown to have antimicrobial activity against *Salmonella enteridis* (Van Immerseel *et al.*, 2004), *Campylobacter jejuni* (Molatová *et al.*, 2010), *Escherichia Coli* (Fortuoso *et al.*, 2019) and *Clostridium perfrigens* (Skřivanová *et al.*, 2005; Timbermont *et al.*, 2010) which are known pathogenic bacteria in quail production. On the other hand, substantially higher inclusion of lauric acid inhibited *Lactobacillus* and *Firmicutes* spp. leading to increased population of *Enterobacteriaceae* spp. (van der Hoeven-Hangoor *et al.*, 2013). The influence of the substitution of conventional vegetable oils (corn oil, soybean oil) by BSF oil on poultry caecal microbiota resulted in reduced bacterial richness, increased *Proteobacteria*, decreased *Bacteroides-*

Prevotella cluster, decreased family *Enterobacteriaceae*, reduced enteric pathogenity (*Listeria monocytogenes*, *Yersinia enterocolitica*, *Pasteurella multocida*) (Chen *et al.*, 2022; Kierończyk *et al.*, 2022; Kierończyk, 2022; Sypniewski *et al.*, 2020), the results however depend on the treatment (partial or total oil replacement) and poultry species.

In this study, we investigated the effect of *H. illucens* larvae oil on caecal microbiota of *Coturnix japonica*.

Commonly used corn oil (CO) was in quail diet partially (25%, 50%) or totally (100%) replaced by BSFL oil and the caecal content was analysed by high-throughput sequencing (HTS) of 16S rRNA fragments and evaluated for the bacterial diversity, community structures and taxonomic composition.

2 Materials and methods

Ethics statement

The animals were treated in accordance with the EC Directive 63/2010/EEC on the protection of the animals used for experimental and other scientific purposes. The experimental procedures were approved by the Ethical Animal Care and Use Committee of the Department of Veterinary Medicine and Animal Production of the University of Napoli Federico II, Italy (prot.N. 2017/0017676). The trial was carried out on a private quail's farm in Sardinia (Italy).

Animals and experimental design

A total of one hundred 7 days old Japanese quails (*Coturnix japonica*) were equally divided in 4 groups (5 replicates of 5 birds/replicate) in galvaniased metal cages (100 cm length × 50 cm depth × 25 cm height), where they were reared until 42 days of age. At the beginning of the trial the quails were housed at 32-35 °C using the infrared heat lamps (150 W) situated at 25 cm from the floor of the cages. Then, the temperature was gradually decreased until the end of fourth week to reach final temperature of 23-24 °C. The lighting programme was 16:8 h light: dark.

Diet

The birds were fed 4 isoproteic and isoenergetic diets, which differed only in the source of fat (oil). The control group (C) was fed a basal diet containing corn oil (CO) and the three experimental groups (BSFO 25, BSFO 50 and BSFO 100) were fed the same diet, in which, CO was partially (25%, 50%) or totally (100%) substituted by BSF oil (BSFO) extracted from BSF larvae by cold press extraction technique, as commercially available from PROTIX, Dongen, the Netherlands to keep the amount of 50 g of added oil per kg of diet. PROTIX, recognised as a leading insect farming company in EU, assure the purity of their industrial products as they must conform with the relevant EU legislations by ensuring high standards of animal welfare in insect production. Feed and fresh water were administered ad libitum. The analysis of the diets' contents was performed according the AOAC (2005) methods: for crude protein (method 978.04), ether extract (method 920.39), crude fiber (method 978.10), dry matter (2001.12) and ash (method 930.05). Based on the composition of the diet's constituents, the content of amino acids was estimated. From the diets' chemical composition, the metabolisable energy (ME) was calculated based on the NRC, (1994) equations. Detailed information about the experimental diets and their nutritional characteristics are shown in the Table 1.

To perform a fatty acids (FAs) transmethylation analysis, a base-catalysed procedure was used, as reported by Christie *et al.* (1982) and modified by Chouinard *et al.* (1999). A gas chromatograph (Agilent technologies, model 5890, Santa Clara, CA, USA), equipped with a fused SP-2560 silica capillary column L × I.D. 100 m × 0.25 mm, df 0.20 μm (Supelco, Inc., Bellefonte, PA, USA) was used for FAs methyl esters quantification. Helium was used as carrier gas, with 280 kPa of constant pressure, 50 mL/min splitting flow, and 1 μL of injection volume. Concerning the column parameters, for 15 min the column temperature was sustained at 170 °C, then increased up to 240 °C by adjustment of 5 °C/min. The overall execution duration was 64 minutes. The FAs peaks were categorised through comparision of the retention times of a commercial standard comprising 37 methyl esters of FAs (Sigma-Aldrich, St. Louis, MO, USA). Controlling of the CLA isomers retention time was done by the elution of the commercial standard (Larodan AB, Solna, Sweden) of these fatty acids. By applying a percentage calculation to the overall area of the eluted peaks, the area of each specific FA found in the sample was determined. The fatty acid profile analysis of *H. illucens* oil, corn oil and the diets used in this experiment are shown in Table 2.

Growth performance

The live weights (LW) of birds were recorded individually at the beginning (day 7) and the end of the experiment (day 42). The amounts of administered feed and leftovers were measured daily to calculate the birds' feed intake. Average daily feed intake (ADFI), average

TABLE 1 Chemical and nutritional composition of experimental diets (as-fed)

Diets	Control	BSFO 25	BSFO 50	BSFO 100
Ingredients g/100 g as fed				
Corn 8.8	48.1	48.1	48.1	48.1
Soybean meal 44	43	43	43	43
BSF oil	0	1.25	2.5	5
Corn Oil	5	3.75	2.5	0
Calcium carbonate	1	1	1	1
Dicalcium phosphate	2	2	2	2
Salt	0.3	0.3	0.3	0.3
Methionine	0.1	0.1	0.1	0.1
[1]Premix Vit-Min	0.5	0.5	0.5	0.5
Chemical composition				
Dry matter (%)	90.2	89.9	90.1	89.8
Ash (%)	6.52	6.43	6.47	6.39
Crude protein (%)	23.1	23.1	23.1	23.1
Crude fiber (%)	9.32	9.15	9.43	9.24
Ether extract (%)	7.73	7.86	8.04	7.92
Lysine (%)	1.41	1.41	1.41	1.41
Calcium (%)	0.96	0.96	0.96	0.96
Phosphorus (%)	0.44	0.44	0.44	0.44
Methionine + Cysteine (%)	0.92	0.92	0.92	0.92
2 ME (kcal/kg)	2,900	2,900	2,900	2,900

1 Provided per kg of product: Fe, 50,000 mg; Co, 200 mg; Cu, 8,500 mg; Mn, 75,000 mg; Zn, 70,000 mg; Se, 250 mg; I, 1,500 mg; folic acid, 500 mg; pantothenic acid, 13.5 g; niacin, 30 g; vit. A, 10,000,000 IU; cholecalciferol 50,000 mg; vit. K3, 4,000 mg; vit. B2, 5,000 mg; vit. B6, 2,000 mg; vit. B12, 10,000 mg; vit. E (dl-a-tocopheryl acetate), 21,978 IU. 2: Metabolisable energy.

daily gain (ADG) of live weight, and feed conversion ratio (FCR) were calculated for the entire experimental period (35 days).

Samples collection

Ten quails (42 days old) were picked from each group (2 birds from each replicate) and slaughtered by cervical dislocation. The intestine was immediately removed from the carcasses and the luminal contents from the caeca were collected, packed and sealed in sterilised micro-centrifuge tubes (2.0 ml, Eppendorf®), immediately refrigerated (for about 1 hour) and transported into laboratory. The samples were frozen at –80 °C and lyophilised using Heto powerdry LL3000 freeze dryer (Thermo Fisher Scientific, Wilmington, DE, USA). The dried samples were transported to the Institute of Animal Physiology and Genetics of the Czech Academy of Sciences (Prague, Czech Republic) for further analysis. The list of samples is summerised in Supplementary Table S1.

DNA extraction

The DNA was extracted from the dry caecal samples using PowerSoil DNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The concentration and quality of the nucleic acids were measured using a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the DNA was stored at –20 °C until further use.

Amplification and purification of 16S rRNA fragments

The DNA isolates were diluted 10-fold in nuclease-free water and 2 µl (~20 ng/µl) were used as templates for the PCR reaction. The V4-V5 region of the 16S rRNA gene was amplified using the specific primer pair, BactB-F (GGA TTA GAT ACC CTG GTA GT) and BactB-R (CAC GAC ACG AGC TGACG) (Fliegerova *et al.*, 2014), using the EliZyme™ HS FAST MIX Red Master Mix (Elisabeth Pharmacon, Brno, Czech Republic). Thermal cycling conditions included a denaturation step for 5 min at 95 °C, followed by 25 cycles of 30 s at 95 °C, 30 s at 57 °C and 30 s at 72 °C, a final elongation step at 72 °C for 5 min. The length and quality of PCR amplicons were checked by 1.5% agarose gel electrophoresis. The amplicons were purified using the Monarch® PCR and DNA Cleanup Kit (New England BioLabs, Ipswich, MA, USA) and their concentration was checked using a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Library preparation and next generation sequencing

The libraries were prepared from purified amplicons using the NEBNext Fast DNA Library Prep Set kit (New England BioLabs, Ipswich, MA, USA) and the Ion Xpress Barcode Adapters 1-96 Kit (Thermo Fisher Scientific, Waltham, MA, USA). The quality of the libraries was checked by the Agilent 2100 Bioanalyser instrument using the Agilent High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA). The quantification of the libraries was done using the KAPA Library Quantification Kit (KAPA Biosystems, Roche, Pleasanton, CA, USA). A volumetric pooling of each library was done after its normalisation by dilution to achieve a 30 pM concentration and used for the template amplification and enrichment by the Ion OneTouch™ 2 instrument using the Ion PGM™ HiQ™ View OT2 Kit-400

TABLE 2 Fatty acid profile of the dietary fats and experimental diets (% total FA)

Fatty acid	Dietary fats		Experimental diets			
	CO	BSFO	Control	BSFO 25	BSFO 50	BSFO100
C4:0	0.15	1.57	0.01	0.03	0.04	0.08
C12:0	–	37.71	–	0.47	0.94	1.89
C14:0	–	10.46	0.08	0.22	0.35	0.61
C16:0	10.85	17.11	11.12	11.20	11.28	11.44
C16:1	–	2.89	–	0.04	0.07	0.15
C18:0	1.51	2.79	2.68	2.70	2.71	2.75
C18:1t9	0.12	0.34	0.006	0.009	0.011	0.017
C18:1 cis-9	30.4	11.97	23.027	22.106	22.566	22.797
C18:2cω6 (LA)	54.9	12.13	52.75	52.21	51.68	50.61
C20:0	0.54	0.21	0.76	0.75	0.75	0.74
C20:1	–	0.04	0.043	0.044	0.044	0.045
C18:3ω3(ALA)	1.07	0.68	4.62	4.62	4.61	4.60
SFA	13.35	70.41	14.84	15.55	16.27	17.69
MUFA	30.56	15.68	23.08	22.89	22.71	22.33
PUFA	56.08	13.51	57.55	57.01	56.48	55.42
ω 6	54.96	12.16	52.75	52.22	51.68	50.61
ω 3	1.12	0.68	4.79	4.79	4.78	4.77
Ratios						
PUFA/SFA	4.20	0.19	3.75	3.70	3.65	3.55
ω 6/ω3	49.26	17.89	50.04	49.65	49.26	48.47
LA/ALA	51.40	17.85	59.65	59.23	58.81	57.97

CO = corn oil fatty acid profile; BSF = black soldier fly; LA = linoleic acid; ALA = alpha linoleic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

(Thermo Fisher Scientific, Waltham, MA, USA). The enriched template was sequenced with the Personal Genome Machine (PGM™) System (Thermo Fisher Scientific, Waltham, MA, USA) using the Ion PGM™ Hi-Q™ View Sequencing solutions kit and the Ion 316™ Chip v2 BC according to the manufacturer's protocols.

Bioinformatic and statistical analysis

The growth performance data were subjected to a one-way ANOVA with diet as a fixed effect, following the GLM procedure (General Linear Model) of the IBM SPSS Statistics package (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA).

The raw sequences retrieved in FASTQ format from the Ion Torrent software were analysed using Qiime2 version 2020.2 software (Bolyen *et al.*, 2019). The sequences were quality filtered, and denoised using DADA2 and chimeras were removed (Callahan *et al.*, 2016). The rarefaction was conducted to ensure a uniform sampling depth, the dataset was subsampled to a minimum of 5000 reads per sample. The rarefaction curves reached a plateau, showing that the depth of sequencing was adequate and all the species in the samples were sufficiently covered (Supplementary Figure S1). The sequences were clustered into Amplicon Sequence Variants (ASVs) by VSEARCH, and the taxonomic assignment was achieved with a BLAST search against the SILVA database (version 132) with a 97% threshold (Rognes *et al.*, 2016). The bacterial diversity was assessed using alpha diversity indices (Faith's Phylogenetic Diversity, Pielou Evenness, and Shannon Entropy), the comparison between the groups was done with the Kruskal-Wallis H test and visualised using the qiime2R, tidyverse and ggplot2 packages in R-Studio (version 4.2.1) (Bisanz, 2018; Wickham, 2016; Yan Hui, 2021). Beta diversity was calculated using the Jaccard distance matrix. The principal coordinate analysis (PCoA) was used for the visualisation, and the resultswere plotted using EMPeror (Vázquez-Baeza *et al.*, 2013). The permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was performed to determine the statistical differences between the groups, PERMDISP test was also performed to check the homogeneity of dispersions among the ani-

mal groups. Linear discriminant analysis (LDA) with an effect size (LEfSe) algorithm (Segata *et al.*, 2011) was done on the Galaxy web module (http://huttenhower.sph.harvard.edu/galaxy/ (accessed on 15 August 2022) to identify the significantly differentially abundant taxa, with the following parameters: alpha = 0.05 and a minimum LDA score = 2.0.

The sequence information was deposited in the Sequence Read Archive under accession number: PRJNA871111.

3 Results

Fatty acid composition of the dietary fat sources

Oil sources (50 g/kg) were added into a soybean meal and corn base diet. The analysis of the experimental diets showed a similar content of crude protein, ether extract and ash, but the fatty acid composition of the oil sources clearly differed as shown in Table 2. BSF larvae oil was rich in saturated fatty acids (SFA) as the result of high concentrations of lauric (C12:0) and myristic (C14:0) acids, while corn oil had higher content of monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) concentrations represented by oleic (C18:1 cis-9) and linoleic acid (C18:2cω6), respectively.

Growth performance

No statistically significant differences ($P > 0.05$) were recorded for the birds' live weights (LW), average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR). Results are summarised in Table 3 showing no influence of the different dietary treatments on the animal growth parameters.

Diversity and similarity of bacterial communities

The bacterial population in the caecal samples of quails of the 4 groups was qualitatively and quantitatively analysed for species richness, evenness and phylogenetic diversity. The analysis showed a lower diversity richness and evenness mainly in the samples of quails fed diet with a total replacement of corn oil by *H. illucens* larvae oil, as shown in the Supplementary Table S2. Mainly, the Shannon entropy revealed a significant difference in richness between the control group and the BSFO 100 group ($P = 0.03$), also between the BSFO 25 group and the BSFO 100 group ($P = 0.03$), as shown in Figure 1A. Similarly, Pielou evenness index showed a significant difference between the control group and the BSFO 100 group ($P = 0.02$), also between the BSFO 50 group and BSFO 100 group ($P = 0.04$), as shown in Figure 1B. However, the Faith's phylogenetic distance showed no significant differences among the groups. The results of the alpha diversity assessment are shown in Supplementary Table S3. The Beta diversity, which evaluates the similarity/differences in bacterial composition among the groups, was assessed using Jaccard's non-phylogenetic distance matrix. A Principal Coordinate Analysis (PCoA) plot (Figure 2) shows the spatial separation of the samples and statistical analysis revealed significant differences among all the studied group (PERMANOVA $P < 0.05$) except the comparison of control group with BSFO 25 group ($P > 0.05$). PERMDISP showed no statistical differences among the groups ($P < 0.05$), indicating a low intergroup variability (Table 4).

Taxonomical composition

In total, the caecal bacterial community consisted of 5 phyla including 110 bacterial phylotypes. *Firmicutes* represented the dominant phylum in all four groups of animals (C: 87.3 ± 6.3%; BSFO 25: 89.1 ± 5%; BSFO 50: 89.4 ± 4.1%; BSFO 100: 91.5 ± 2.8%) followed by a minor population of *Actinobacteria*, *Tenericutes*, *Proteobacteria* and *Bacteroidetes*. Regardless of the quails' diet, the major order of *Firmicutes* was *Clostridiales* mainly represented at the family level by *Ruminococcaceae* and *Lachnospiraceae*. The second most abundant order was *Lactobacillales*, which was mainly represented at the family level by *Streptococcaceae* and *Lactobacillaceae*, as shown in Figure 3A. At genus level, the most abundant genera were *Subdoligranulum*, unclassified genus within family *Lachnospiraceae*, *Blautia* and *Clostridiales bacterium* CHKCI001, forming together more than half of the sequences in all four groups of quails. Less abundant genera with a meaningful relative abundance (>1%) were unclassified genus within family *Lachnospiraceae*, *Eubacterium hallii* group, *Ruminococcus* torques group, *Ruminococcaceae* UCG-014, *Lactobacillus*, *Butyricicoccus*, *Sellimonas*, unclassified genus within family *Ruminococcaceae*, *Ruminococcaceae* UCG-005. Bacterial taxa with relative abundance lower than 1% are summarised as 'Others' in Figure 3 and listed in the Supplementary Table S4.

Determination of taxonomic biomarkers

Linear discriminant analysis effect size (LEfSe) was performed to determine the bacterial taxa with significantly different levels of abundance between the control group and the other groups of quails in which the *H. illucens* larvae oil was included in diet. The analysis comparing the control group C to BSFO 25 resulted

TABLE 3 Effect of the dietary inclusion of *Hermetia illucens* larvae oil on the growth performance of broiler Japanese quails

Item	Experimental Diets				*P*-value	SEM
	Control	BSFO 25	BSFO 50	BSFO100		
IBW (g,d 7)	36.04	35.83	35.44	36.7	0.615	0.36
FBW (g,d 42)	221.64	224.7	223.4	225.8	0.927	2.4
ADFI (g)	16.67	16.58	16.6	16.61	0.388	0.02
ADG (g)	5.3	5.4	5.37	5.4	0.949	0.07
FCR (g/g)	3.19	3.14	3.1	3.12	0.831	0.03

BSFO = black soldier fly oil; IBW = initial body weight; FBW = final body weight; ADFI = average daily feed intake; ADG = average daily gain; FCR = feed conversion ratio; SEM = standard error of the mean.

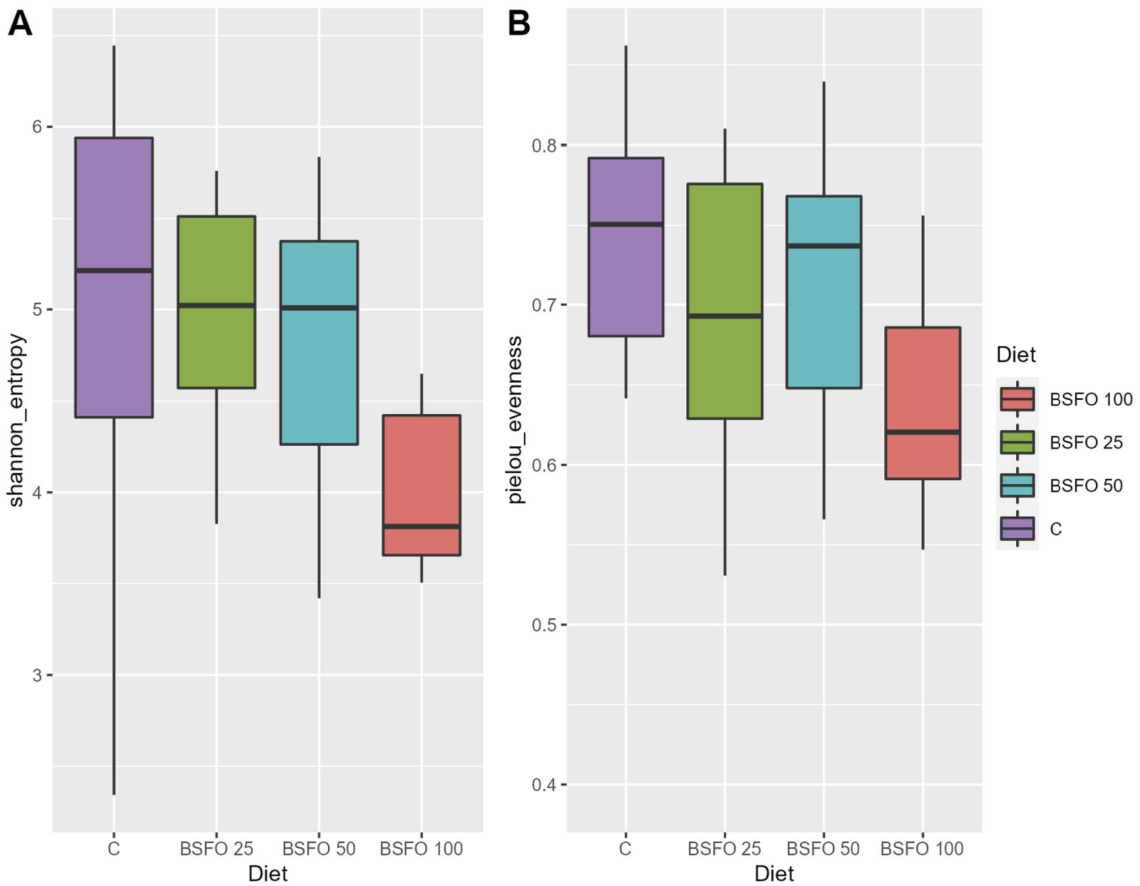

FIGURE 1 Comparison of diversity indices for caecal bacterial communities of four groups of quails fed different diets. (A) The bacterial diversity estimated by the Shannon entropy. (B) The bacterial evenness estimated by Pielou evenness index. The Kruskal–Wallis pairwise test was used for sample comparison.

in 5 differentially abundant bacterial taxa (LDA score > 2.0). Only 1 taxon, *Anaerotruncus* (Family *Ruminococcaceae*), had significantly higher relative abundance in the control group (green bars) and 4 taxa had significantly higher relative abundance in the BFSO 25 group (red bars), including *Fusicatenibacter* (family *Lachnospiraceae*), *Lactobacillaceae*, *Lactobacillus* and *Eubacterium hallii* group (Family *Lachnospiraceae*) (Figure 4A,D). The comparison of the control group C with BSFO 50 showed 6 differentially abundant bacterial taxa (LDA score > 2.0). Five taxa had significantly higher relative abundance in the control group (green bars), including 3 phylotypes of bacilli (*Bacillales*, *Bacillaceae* and *Bacillus*), *Anaerotruncus* (Family *Ruminococcaceae*) and *Coprococcus* 3 (family *Lachnospiraceae*). Only 1 taxon, *Fusicatenibacter* (family *Lachnospiraceae*), had significantly higher relative abundance in the BSFO 50 group (red bars) (Figure 4B,E). LEfSe analysis compar-

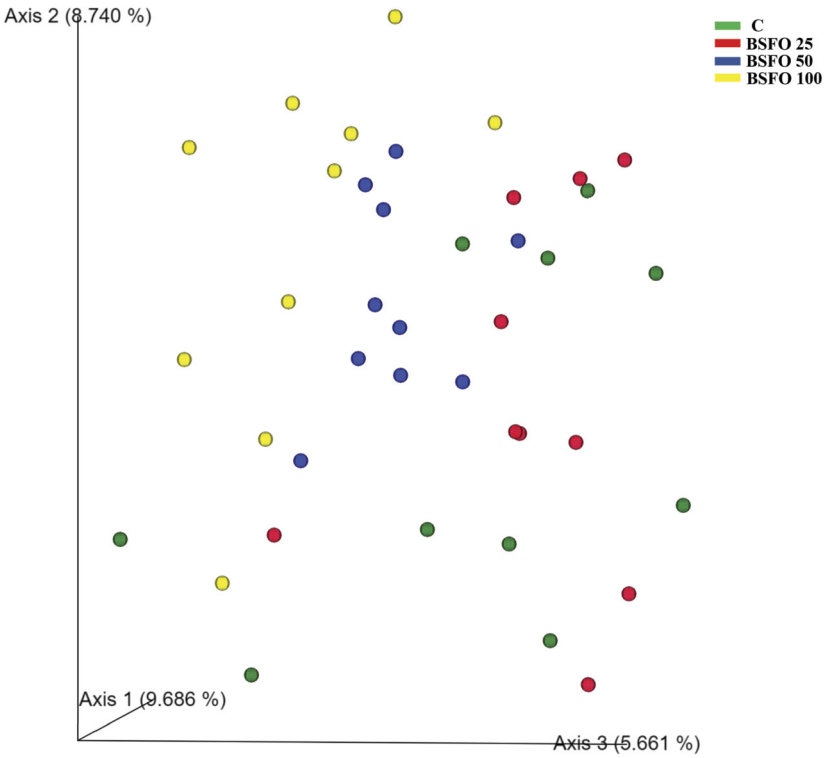

FIGURE 2 Principal Coordinate Analysis (PCoA) showing the Jaccard's distance matrix between caecal bacterial community compositions of four groups of quails fed on different diets. Each dot represents one sample. The percentage of variation explained by the plotted principal coordinates is indicated on the axes.

TABLE 4 Permutational multivariate analysis of variance (PERMANOVA) and dispersions (PERMDISP) based on Jaccard distance matrix)

Diet	Jaccard distance matrix	
	PERMANOVA *P*-value	PERMDISP *P*-value
Control vs BSFO 25	0.092	0.699
Control vs BSFO 50	0.003*	0.068
Control vs BSFO 100	0.004*	0.413
BSFO 25 vs BSFO 50	0.002*	0.176
BSFO 25 vs BSFO 100	0.001*	0.736
BSFO 50 vs BSFO 100	0.004*	0.261

* Significant difference ($P < 0.05$).

ing the control group C to BFSO 100 resulted in the highest number of taxa with significantly different relative abundances. A total of 16 differentially abundant bacteria (LDA score > 2.0) were found. Fourteen taxa had significantly higher relative abundance in control group C (green bars) and only 2 were significantly more abundant in the BSFO 100 group (red bars). In the control group C, the increased phylum Actinobacteria (LDA score > 3.0) included several coriobacterial phylotypes (*Coriobacteriia*, *Coriobacteriales* and *Eggerthellaceae*). Increased class *Erysipelotrichia* included several *Erysipelotrichales* phylotypes. The genera *Eubacterium* brachy group (Family XIII), *Anaerotruncus* and *Negativibacillus* (belonging to *Ruminococcaceae* family), *Coprococcus 3*, *Anaerostipes* and *Lachnoclostridium* (belonging to *Lachnospiraceae* family) and finally *Bacillus* (family *Bacillaceae*) were also significantly higher in the control group. On the other hand, in the BSFO 100 group, the genera *Fusicatenibacter* (family *Lachnospiraceae*) and *Subdoligranulum* (family *Ruminococcaceae*) were significantly enriched (LDA score > 3.6) (Figure 4C,F).

4 Discussion

In this study, we examined the influence of black soldier fly larvae oil (BSFO) on bacterial community of the caecal content of Japanese quails. BSFO has attracted attention in the recent years as an alternative oil source in poultry nutrition, because this insect oil, which is rich in medium-chain fatty acids (MCFA), especially lauric acid (Ewald *et al.*, 2020), could have a positive effect on growth performance, gut health and meat quality in broiler chickens (Cullere *et al.*, 2019; Kim *et al.*, 2020; Schiavone *et al.*, 2018; van Immerseel *et al.*, 2006). BSFL

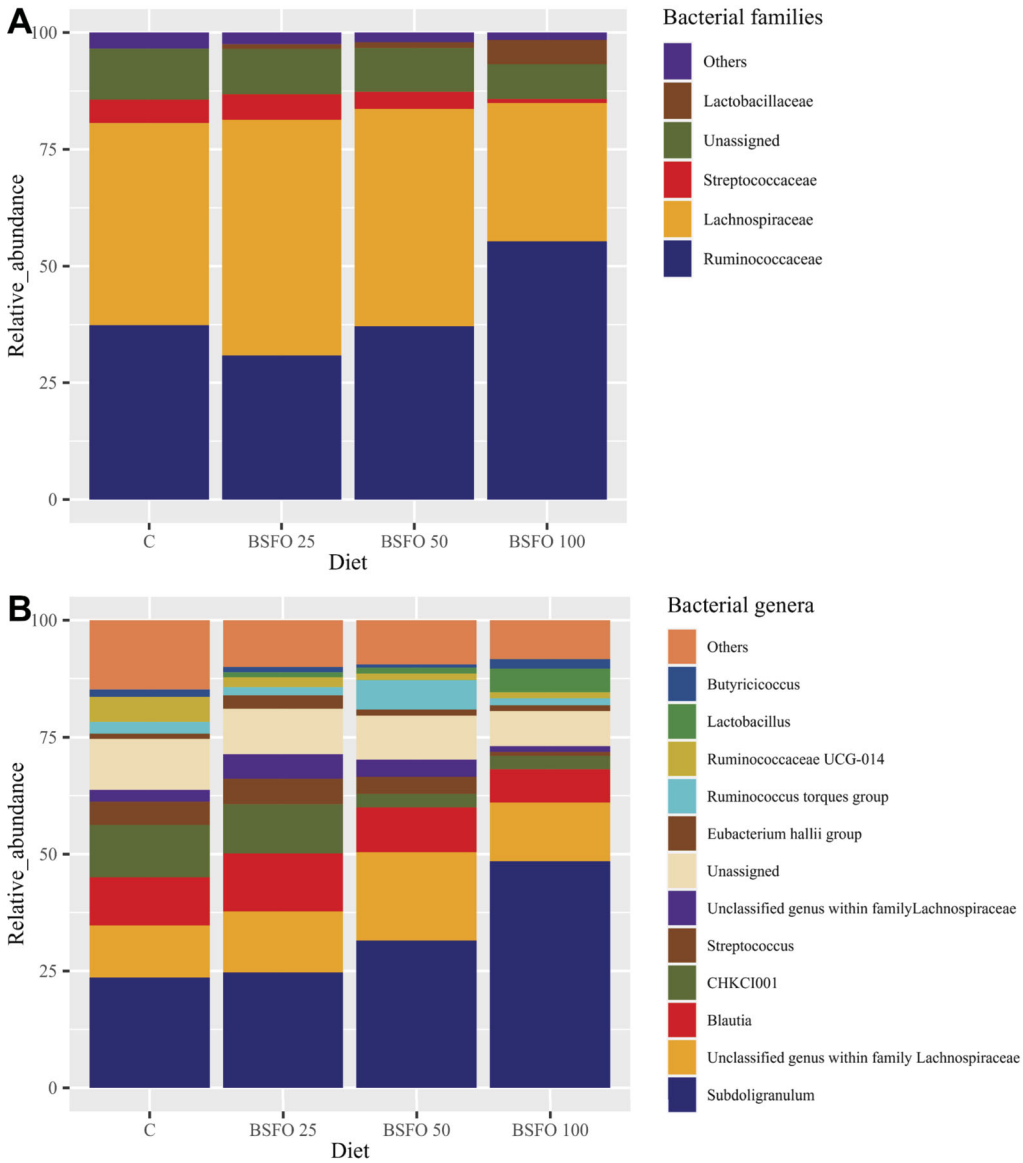

FIGURE 3 Relative abundance of caecal bacteria in four groups of quails fed different diets illustrated at family (A) and genus level(B). Taxa with a relative abundance lower than 1% are grouped as 'Others'.

and its oil moreover exhibit antioxidant, antimicrobial and immune-modulating properties (Lee *et al.*, 2018; Mlcek *et al.*, 2014). The research on the effect of the insect oil on poultry is still scarce and insufficient and completely lacking on quails. Therefore, we focused on the study of the influence of BSFO on the caecal microbiota of Japanese quails. Even if the whole poultry digestive tract is important and each part plays a specific role in the digestion process and absorption of nutrients, the caecum represents the primary site of fermentation in the avian GIT responsible for transformation of indigestible carbohydrates (cellulose, starch) into short-chain fatty acids (SCFA) (Józefiak *et al.*, 2004), whose processes are closely related to productivity (Díaz Carrasco *et al.*, 2018; Waite and Taylor, 2014). With up to 10^{11} cells per gram, caeca have the greatest bacterial number and biodiversity along the poultry GIT (Grant *et al.*, 2018) and it is dominated by the phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria* (Oakley *et al.*, 2014).

The dietary treatments used in this study did not significantly affect the animals growth performance parameters (ADFI, ADG and FCR). Results in respective poultry literature regarding an influence of insect oil on feed intake and body weight gain are not consistent describing positive (Benzertiha *et al.*, 2019a), or, more often, no significant effects on growth of broiler chickens (Benzertiha, *et al.*, 2019b; Chen *et al.*, 2022; Kawasaki *et al.*, 2019; Kim *et al.*, 2020; Schäfer *et al.*, 2023), which is in agreement with our study. The discrepancies can be attributed to different experimental

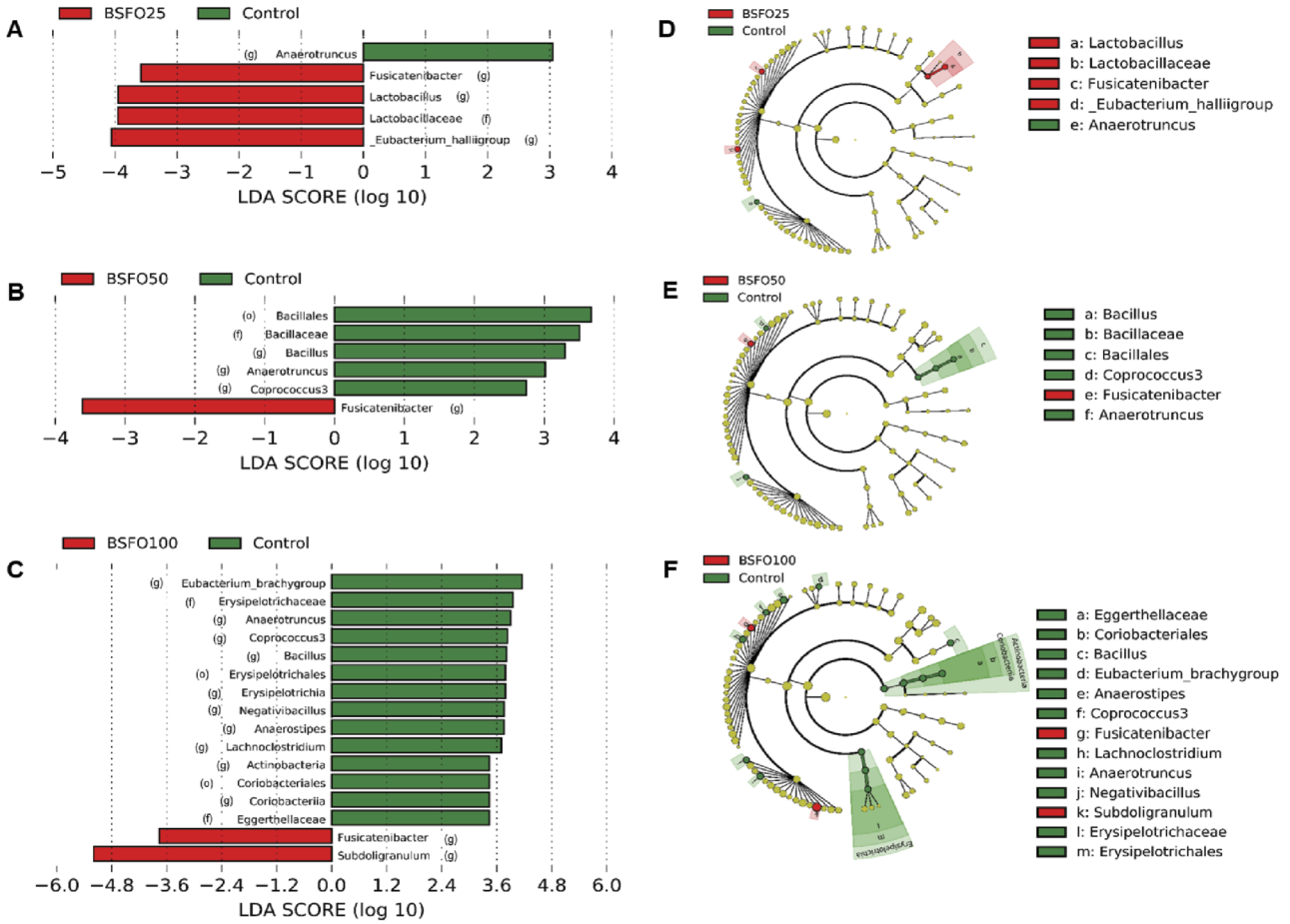

FIGURE 4 Linear discriminant analysis (LDA) scores on different taxonomical levels of four groups of quails fed on different diets: phylum(p), class(c), order(o), family(f), genus(g). (A), (B) and (C) represent the histogram plots of LDA scores for differentially abundant taxa among the groups. The length of the bar represents the log10 transformed LDA score, indicated by vertical dotted lines. Positive LDA scores (green bars) and negative LDA scores (red bars) represent bacterial taxa over-abundant in the corresponding group. (D), (E) and (F) represent the cladograms showing the phylogenetic relationship among different groups of organisms with significantly different levels of abundance.

conditions, variations in animal age, breed, trial settings and management, feed composition, and the wide range in FA contents of insect oils. However, a broader view of the influence of BSF oil as part of the poultry diet indicates the beneficial effect on the animal health and production (Gasco *et al.*, 2018).

Regarding the influence of the diet on the diversity of the caecal microbiota, the richness and equity in bacterial species abundance was significantly lower in the BSFO 100 group compared to the Control group. The Principal Coordinate Analysis (PCoA) based on the Jaccard distance matrix, showed the spatial separation of the samples, with statistical differences between the studied groups. This is in agreement with Biasato *et al.* (2020) who showed a lower alpha diversity in broiler chicken fed 15% *H. illucens* meal and a significant beta diversity (using the weighted UniFrac distance matrix) between birds fed *H. illucens* diets and control diet fed a maize, corn gluten and soybean meals (Biasato *et al.*, 2020). In contrast, Dabbou *et al.* (2021) study didn't find differences in alpha diversity but the Principal component analysis (PCA) based on OTUs relative abundance showed a clear separation of the control samples fed with soybean oil from sample of the BSF oil group diet. The significant beta diversity differences were also described in several other insect-fed poultry studies (Biasato *et al.*, 2018, 2019; Borrelli *et al.*, 2017; Dabbou *et al.*, 2021). Our results are partially in agreement with Chen *et al.* (2022) who also found a lower richness in alpha diversity in the chickens fed different levels of BSF oil, however the PCoA based on OTUs revealed no significant difference in beta diversity between the studied groups. A decreased bacterial diversity is in general a negative phenomenon because the species diversity plays an important role in maintaining the stability of the intestinal ecosystem and its normal ecological function. A loss of diversity could initiate immune-mediated disorders, which can result in

the development of inflammatory disease. This is well documented mainly in human research (Al Bander *et al.*, 2020) but also in poultry science (Wickramasuriya *et al.*, 2022; Yang *et al.*, 2022). In poultry research the GIT bacterial species diversity is considered a key factor of pathogen exclusion (Pedroso *et al.*, 2021).

In this work, the bacterial community composition in the quail caecal samples was also dominated by *Firmicutes*, regardless of the type of diet, which is in agreement with the previous studies on quails (Du *et al.*, 2020; Su *et al.*, 2014; Wilkinson *et al.*, 2016) and other poultry (Andreani *et al.*, 2020; Biasato *et al.*, 2020; Cardenas *et al.*, 2021; Costa *et al.*, 2017; Danzeisen *et al.*, 2011; Józefiak *et al.*, 2020; Moula *et al.*, 2018; Wei *et al.*, 2013; Yeoman *et al.*, 2012; Zou *et al.*, 2018). At the family level, the dominance of the families *Ruminococcaceae* and *Lachnospiraceae* (>80% in all treatment groups) in the caecal microbiota is in agreement with many previous studies, (Andreani *et al.*, 2020; Biasato *et al.*, 2020; Danzeisen *et al.*, 2011; Józefiak *et al.*, 2020; Moula *et al.*, 2018; Wei *et al.*, 2013; Wilkinson *et al.*, 2016). These two families from the order *Clostridiales* are highly specialised for the degradation of complex plant material and have the considerable capacity to break down a full range of plant-derived substrates including cellulose, hemicellulose and starch (Biddle *et al.*, 2013; Broom, 2018; Stanley *et al.*, 2016; Yang *et al.*, 2017). Their production of SCFA (mainly acetate, butyrate and propionate) improves feed efficiency and butyrate supports gut health. The production of butyrate is an essential energy source for colonocytes (Jung *et al.*, 2015), it ameliorates the integrity of tight-junctions (Peng *et al.*, 2009) and has an anti-inflammatory effect or role by reducing the inflammatory response (Furusawa *et al.*, 2013). The two dominant families, *Ruminococcaceae* and *Lachnospiraceae*, however, responded differently to the inclusion of different levels of black soldier fly larvae oil in the quail diet. In BSFO 25 group the *Ruminococcaceae* decreased (30.9%) and *Lachnospiraceae* increased (50.5%), while in BSFO 100 the shift was the opposite with *Ruminococcaceae* forming (55.3%). In addition, an increase in *Lactobacillaceae* and a decrease in *Streptococcaceae* was observed in the BSFO 100 group. *Lactobacillaceae* are known to be beneficial in improving the intestinal health and growth performance of poultry (Chateau *et al.*, 1993; Yan *et al.*, 2017). While *Streptococcaceae* is a source of streptococcosis in poultry and its reduction thus can be considered as the beneficial effect of HI oil inclusion. On the other hand, regardless of the diet, the abundance of *Clostridiaceae* and *Bacteroidaceae* families was found to be low, which is in disagreement with previous studies on quails (Du *et al.*, 2020) and chickens (Biasato *et al.*, 2020; Cardenas *et al.*, 2021).

At the genus level, the core bacterial genera were *Subdoligranulum*, Unclassified genus within family *Lachnospiraceae*, *Blautia*, and *Clostridiales bacterium* CHKCI001, which represented together 56.3% of the sequences in the control group, 60.7% in the BSFO 25 group, 63% in the BSFO 50 group and 71% in the BSFO 100. This is in good agreement with previous studies describing a predominance of these taxa in healthy broiler chickens (Biasato *et al.*, 2020; Cardenas *et al.*, 2021; Clavijo and Flórez, 2018; Emami *et al.*, 2021; Hou *et al.*, 2016; Ijaz *et al.*, 2018; Kong *et al.*, 2021; Polansky *et al.*, 2016). The gradual effect of increasing doses of black soldier fly larvae oil is well seen at the genus level (Figure 3B), which is evident in particular by the increasing amount of *Subdoligranulum sp*. This genus belongs to the *Ruminococcaceae* family and is a butyrate-producing organism closely related to the *Faecalibacterium* genus. *Subdoligranulum* has been linked to multiple beneficial health effects on host energy metabolism and several interesting findings supported using this organism as promising probiotic (van Hul *et al.*, 2020). This genus promotes the development of intestinal epithelial cells, which can minimise *Salmonella* invasion and colonisation (Eeckhaut *et al.*, 2008). Danzeisen *et al.* (2011) stated that the use of antibiotics in poultry feed increased the prevalence of *Subdoligranulum*, leading to an increased approval of the hypothesis that the inclusion of BSF oil can be a substitute for conventional antibiotic usage, probably due to the high lauric acid content in its fatty acid composition (Boyen *et al.*, 2008). With respect to other highly abundant genera, *Blautia* was increased in BSFO 25 and unclassified genus within family *Lachnospiraceae* was increased in BSFO 50. *Clostridiales bacterium* CHKCI001 and *Streptococcus* were suppressed in BSFO 50 and 100. With respect to less abundant genera, the *Ruminococcus torques* group was increased in the BSFO 50 group and *Lactobacillus* was elevated in the BSFO 100 group. Both these two bacteria are supposed to have positive effect. The *Ruminococcus torques* has been shown to be more abundant in broiler chickens fed a diet with a blend of medium-chain fatty acids (MCFA) (Kers *et al.*, 2019) it and has been associated with improved performance in the broiler caeca (Torok *et al.*, 2011). *Lactobacillus* was identified as characteristic OTU of the caecal microbiota of broiler chickens fed 10% of *H. illucens* meal inclusion (Biasato *et al.*, 2020) and in general it is well known for its positive effect on gut health and the immune cells

homeostasis (Ren *et al.*, 2016; van Tassell and Miller, 2011).

The LEfSE analysis identified taxa with significantly different abundances in each animal group. The increased relative abundances of *Anaerotruncus* (family *Ruminococcaceae*) in the control group and *Fusicatenibacter* (family *Lachnospiraceae*) in all three groups including *H. illucens* oil were the common features resulting from all three analyses. *Anaerotruncus* may enhance the absorption of volatile fatty acids to increase the energy utilisation of recipients, leading to an increased muscle fiber diameter and decreased drip loss (Lei *et al.*, 2022). *Fusicatenibacter* is SCFA-producing taxon which produce formate and acetate and it can protect the equilibrium of the intestinal microbial community (Qiu *et al.*, 2020; Takada *et al.*, 2013), suppresses the intestinal inflammation (Takeshita *et al.*, 2016), reduces diarrheal symptoms (Yang *et al.*, 2021) and regulates colonic motility (Zhang *et al.*, 2021). As well, *Subdoligranulum*, which was the dominant genus among the four groups and was significantly enriched in the BSFO 100 group compared to the control group (LDA > 4.8), can have positive outcomes as discussed above. The other taxa significantly increased in BSFO 25 group, compared to Control, also can be considered beneficial. The *Eubacterium halii group* (family *Lachnospiraceae*) includes only uncultured bacteria, but *Eubacterium hallii* itself is a butyrate producing organism important for intestinal metabolic balance (Duncan *et al.*, 2004; Engels *et al.*, 2016). *Lactobacillus* genus also have positive effects on the animal health and performance by producing antimicrobial substances (Oakley *et al.*, 2014), short chain fatty acids, exopolysaccharides and additional sources of energy (Pajarillo *et al.*, 2015). The effect however highly depends on species (Brisbin *et al.*, 2015). The comparison of bacterial shift in BSFO 50 and Control group however seems to be more beneficial to the control group. The increased abundance of *Bacilli* phylotypes (*Bacillales*, *Bacillaceae* and *Bacillus*) and butyrate-producing *Coprococcus3* genus in could have a positive impact on nutrient absorption (Aliakbarpour *et al.*, 2012). Similar conclusions could be partially assessed for the compariosn of BSFO 100 group with its respective Control group. Taxa *Eubacterium brachy group*, *Lachnoclostridium* and *Anaerostipes* are butyrate producers (Pryde *et al.*, 2002; Ríos-Covián *et al.*, 2016) and Coriobacterial phylotypes (*Coriobacteriia*, *Coriobacteriales* and *Eggerthellaceae*) are involved in the conversion of bile salts and steroids as well as the activation of dietary polyphenols (Clavel *et al.*, 2014). On the other hand, the increase of *Erysipelotrichales* phylotypes in control group seems to be negative, because they were shown to be associated with lipid metabolism and inflammation (Kaakoush, 2015). From this point of view the replacing corn oil with 25% of *H. illucens* oil would be the best dietary recommendation.

Quails are an economically advantageous type of poultry with growing popularity among consumers and an increasing interest in their research can be expected. Our work on the effect of insect oil on the caecal microbiota proves that the diet is rightly considered to be the most influential factor on the composition of the gut microbiota. The GI tract has the most extensive exposed surface in the body and a wide variety of factors associated with diet can positively or negatively affect the delicate balance among the components of the poultry gut. Therefore the research in this area is of great importance. The understanding of the efficient conversion of feed into its basic components for optimal nutrient absorption is vital for both broiler production and welfare and for broiler breeder.

5 Conclusion

To best of our knowledge, this is the first study evaluating the effect of *H. illucens* larvae oil (BSFL oil) inclusion in quails' diet on the caecal bacterial diversity and composition. The results suggest that insect larvae oil can produce changes in the caecal microbiota by enhancing bacterial genera known for their positive effect on gut health. However, the total replacement of corn oil in quails' diet needs to be further studied, due to the adverse effects on bacterial richness and evenness. Based on our results the substitution of corn oil by 25% of BSF oil could be suggested and adopted in quails' diet due to an increase in beneficial bacteria without any significant alteration in richness or evenness of caecal bacterial microbiota and growth performance. However more studies about other parameters, especially regarding meat quality, are necessary to perform to support this hypothesis. The use of BSFL oil for the manipulation of intestinal microbiota, thus should be applied with caution and more research is required to better understand the potential effects of insect oil on intestinal bacteria.

Supplementary Material

Supplementary material is available online at:
https://doi.org/10.6084/m9.figshare.23999712

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The data presented in this study are openly available in the Sequence Read Archive under the accession number PRJNA871111.

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