Poultry Science®

# Effect of replacing soybean meal with *Hermetia illucens* meal on cecal microbiota, liver transcriptome, and plasma metabolome of broilers

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**ABSTRACT** Despite the existence of a number of studies investigating the effect of insect meal on the growth performance of broilers, knowledge about the metabolic effects of insect meal in broilers is still scarce. Thus, the present study investigated the effect of partial replacement of soybean meal with *Hermetia illucens* (**HI**) larvae meal on the liver transcriptome, the plasma metabolome, and the cecal microbiota in broilers. For the study, 72 male one-day-old Cobb 500 broilers were divided into three groups and fed 3 different diets with either 0% (HI0), 7.5% (HI7.5), or 15% (HI15) defatted HI meal for 35 d. Each group consisted of 6 cages (replicates) with 4 broilers/cage. While body weight (**BW**) gain, feed intake, and feed:gain ratio did not differ between groups, breast muscle weight, carcass yield, and apparent ileal digestibility (**AID**) of 5 amino acids were higher in group HI15 than in group HI0 ($P < 0.05$). Indicators of $\alpha$-diversity (Chao1 and Observed) in the cecal digesta were higher in groups HI15 and HI7.5 than in group HI0 ($P < 0.05$). The abundance of 5 families and 18 genera, all of which belonged to the Firmicutes phylum, in the cecal digesta differed among groups ($P < 0.05$). Concentrations of butyric acid, valeric acid, and isobutyric acid in the cecal digesta were lower in group HI15 than in the other 2 groups ($P < 0.05$), whereas those of total and other short-chain fatty acids were not different between groups. Liver transcriptomics revealed a total of 70 and 61 differentially expressed transcripts between groups HI15 vs. HI0 and between groups HI7.5 vs. HI0, respectively, ($P < 0.05$). Targeted metabolomics identified 138 metabolites, most of which were triglyceride species, being different between the 3 groups (FDR < 0.05). According to this study, dietary inclusion of HI larvae meal has no detrimental impact but increases breast muscle weight and carcass weight in broilers suggesting that HI larvae meal can be recommended as a sustainable alternative protein source for broilers.

**Key words:** broiler, liver transcriptome, plasma metabolome, insect meal, cecal microbiota

2024 Poultry Science 103:103635
https://doi.org/10.1016/j.psj.2024.103635

## INTRODUCTION

Despite the urgent need to increase feed production for monogastric farm animals, such as poultry, this challenge is complicated by the fact that natural resources are becoming increasingly limited due to the rapid growth of the world population and climate protection strategies, such as the European Green Deal aiming to reduce greenhouse gas emissions, have to be considered. Thus, there is a need for alternative feed sources, which are produced in a resource-efficient and sustainable manner. In this regard, protein-rich insect biomass (40−60% protein of dry matter (**DM**) according to Makkar et al. (2014)), which can be sustainably produced by breeding suitable insect larvae like *Hermetia illucens* (**HI**) and *Tenebrio molitor* (**TM**) larvae on different low-value agro-industrial sidestreams (Lienhard et al., 2023; Montalbán et al., 2023), might fulfill these prerequisites. Such a recycling-based production strategy not only protects natural resources and critical ecosystems required for the production of conventional protein sources, such as soybeans, but also reduces competition for food between humans and animals, because agro-industrial sidestreams can be used as food for humans only to a very limited extent. Due to these advantages and the abovementioned climate protection strategies,



Received January 16, 2024.
Accepted March 5, 2024.

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insect meal from HI and TM larvae has been permitted as feed for monogastric farm animals (European Commission, 2021).

To date, a substantial number of studies exists investigating the effect of replacement of soybean meal with whole or partially defatted insect meal from HI or TM larvae in the diets of broilers but most of these studies have focused mainly on the effect on growth performance, the digestibility of nutrients and the microbiome (Borrelli et al., 2017; Schiavone et al., 2019; Attivi et al., 2020; Biasato et al., 2020). In contrast, the effect of these alternative protein sources on the intermediary metabolism of broilers was either not or only marginally addressed. For instance, only few studies have studied selected parameters related to the intermediary metabolism of boilers (Biasato et al., 2018a; Dabbou et al., 2018; Benzertiha et al., 2020). Despite the latter studies demonstrating no negative effects of insect meal on metabolic parameters in broilers, the parameters addressed were either not tissue-specific (e.g., alanine aminotransferase, lactate dehydrogenase) or relatively superficial (e.g., organ indices, histological organ features, blood lipids, blood glucose) and do not allow to draw firm conclusions about the effect of insect meal on intermediary metabolism of broilers. None of the studies with broilers published so far have applied comprehensive screening technologies, such as transcriptomics or metabolomics, which is a prerequisite to evaluate the overall impact of insect meal on intermediary metabolism and to exclude potential risks associated with feeding of insect meal for both broilers and consumers ingesting broiler meat. Thus, in order to close this gap of knowledge and to gain a deeper understanding of the metabolic effects of insect meal in broilers, the present study investigated the impact of isonitrogenous and isocaloric replacement of soybean meal with HI larvae meal in broilers using liver transcriptomics, plasma metabolomics, and cecal microbiota analysis.

## MATERIALS AND METHODS

### Animals and Diets

The feeding trial was approved by the Animal Welfare Office of the Justus-Liebig-University Giessen (approval no. JLU-Nr. 806_M). All experimental procedures described followed established guidelines for the care and handling of laboratory animals. The experiment included 72 male, one-day-old broiler chickens (Cobb 500, Cobb, Wiedemar, Germany) which were assigned to three treatment groups with similar initial body weights (**BW**). Each treatment group consisted of 6 cages (replicates/diet) with 4 broilers/cage. The 2.1 $m^2$-cages were equipped with feed automates and nipple drinkers providing free access to feed and water. The floor of the cages was lined with a cardboard, which was covered with litter to allow pecking, scratching and dustbathing. The cardboards and litter were changed twice per week in the first 2 wk and every 2 d for the last 3 wk. The light regime and the room temperature followed the recommendation of the breeder (Cobb-Vantress, 2018a). The light intensity was 40 Lux and the light regime was (light:dark) 24 h:0 h, 23 h:1 h, 22 h:2 h, 21 h:3 h, 20 h:4 , 19 h:5 h for d 1, 2, 3, 4, 5, 6 and 18 h:6 h from d 7 to 35. The room temperature was 33°C on d 1, measured at pen height, and decreased to 19°C on d 35. The temperature at the cage floor was adjusted with infrared lamps (Albert Kerbl GmbH, Buchbach, Germany) during the first 6 d. The 3 groups (HI0, HI7.5, HI15) were fed three different diets, which contained different inclusion levels of a commercially available partially defatted HI larvae meal (Madebymade, Pegau, Germany) as protein source, in a 3-phase feeding system (starter period: d 1-10, grower period: d 11−21, finisher period: d 22−35). The concentration of the HI larvae meal in the diets HI0, HI7.5, and HI15 was 0, 75, and 150 g/kg diet, respectively. HI larvae meal was included in the diets at the expense of soybean extraction meal, soybean oil, and wheat. The reduction of the amounts of wheat and soybean oil was necessary due to the lower protein content and the higher fat content of the HI larvae meal in comparison with soybean meal. The amino acid concentrations of the diets were adjusted with synthetic amino acids to reach the recommended concentrations of digestible essential amino acids (Cobb-Vantress, 2018b). The composition of the diets is shown in Table 1. The finisher diets contained 0.5% $TiO_2$ in order to determine the apparent ileal digestibility (**AID**) coefficient for amino acids by the indicator method (Short et al., 1996).

### Growth Performance

Individual BW of the broilers were recorded on d 1, 10, 21 and 35. The feed intake of each cage was recorded daily, and the feed:gain ratio was calculated from feed intake and BW gains on cage basis. Carcass yield was calculated as the percentage of the eviscerated carcass weight (final body weight minus weights of head, visceral organs and feet) of final body weight at slaughter.

### Chemical Analysis of Diet Components and Diets

Official methods were used to determine DM, crude protein (**CP**), crude ash (**CA**), ether extract (**EE**), crude fiber (**CF**), and amino acids in the feed components and the experimental diets (VDLUFA, 2012). The protein content of the HI larvae meal was calculated with the published specific *N*-to-protein conversion factor of 4.67 for HI larvae considering the high *N* content of chitin (Janssen et al., 2017). Own calculation of the *N*-to-protein conversion factor of the HI larvae meal based on the analyzed concentrations of amino acids and *N* according to Smets et al. (2021) resulted in a quite similar *N*-to-protein conversion factor. The calculation of the CP content in the diets with HI larvae meal was carried out with a chitin-corrected *N* quantity as the use of an *N*-to-protein conversion factor of 6.25 causes an

**Table 1.** Composition of the broiler diets.

| | Starter | | | Grower | | | Finisher | | |
|---|---|---|---|---|---|---|---|---|---|
| | HI0 | HI7.5 | HI15 | HI0 | HI7.5 | HI15 | HI0 | HI7.5 | HI15 |
| Component, g/kg | | | | | | | | | |
| Maize | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 29.5 | 30.0 |
| Soybean meal | 38.0 | 31.60 | 25.12 | 30.00 | 23.60 | 17.23 | 29.00 | 22.50 | 15.90 |
| Wheat | 19.60 | 19.28 | 19.00 | 27.39 | 27.06 | 26.75 | 28.92 | 29.13 | 28.49 |
| HI larvae meal (defatted) | - | 7.5 | 15.0 | - | 7.5 | 15.0 | - | 7.5 | 15.0 |
| Mineral/vitamin mix* | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Soybean oil | 6.00 | 5.33 | 4.66 | 6.00 | 5.33 | 4.65 | 6.00 | 5.34 | 4.67 |
| Monocalcium phosphate | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 0.90 | 0.95 | 0.90 |
| Calcium carbonate | 1.55 | 1.55 | 1.55 | 1.55 | 1.55 | 1.55 | 1.50 | 1.50 | 1.50 |
| Sodium chloride | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| DL-Methionine | 0.31 | 0.33 | 0.34 | 0.31 | 0.32 | 0.33 | 0.26 | 0.28 | 0.29 |
| L-Threonine | 0.11 | 0.10 | 0.10 | 0.12 | 0.12 | 0.10 | 0.06 | 0.06 | 0.05 |
| L-Lysine | 0.37 | 0.35 | 0.33 | 0.44 | 0.42 | 0.39 | 0.34 | 0.31 | 0.30 |
| L-Valine | 0.16 | 0.06 | - | 0.19 | 0.11 | 0.02 | 0.11 | 0.03 | - |
| L-Isoleucine | - | - | - | 0.04 | 0.03 | 0.02 | 0.01 | - | - |
| L-Arginine | - | - | - | 0.06 | 0.06 | 0.06 | - | - | - |
| $TiO_2$ | - | - | - | - | - | - | 0.5 | 0.5 | 0.5 |

*The mineral/vitamin mix supplied the diets with the following minerals and vitamins per kg (starter/grower/finisher): I 1/1/1 mg; Cu 15/15/15 mg; Mn 100/100/100 mg; Se 0.35/0.35/0.35 mg; Zn 100/100/100 mg; Fe 40/40/40 mg; vitamin A 10.000/10.000/10.000 IU; vitamin $D_3$ 5.000/5.000/5.000 IU; vitamin $K_3$ 3/3/3 mg; vitamin E 80/50/50 IU; vitamin $B_1$ 3/2/2 mg; vitamin $B_2$ 9/8/6 mg; vitamin $B_6$ 4/3/3 mg; vitamin $B_{12}$ 0.02/0.015/0.015; biotin 0.2/0.18/0.18 mg; folic acid 2/2/1.5 mg; nicotinic acid 60/50/50 mg; pantothenic acid 15/12/10 mg; choline chloride 500/400/350 mg.

overestimation and of 4.67 an underestimation of the protein content. For this, the chitin content of the HI larvae meal was measured with a modified method for glucosamine determination according to (Tsuji et al., 1969) as described in more detail (Maheshwari et al., 2020), and the corresponding *N* amount calculated. The concentration of chitin in the HI larvae meal was 14.1% (of DM) equating to 0.97% *N*. The *N* content of the chitin was subtracted from the total *N* content and the CP content of the diets was then calculated with the *N*-to-protein conversion factor of 6.25. The HI larvae meal contained 43.1% (DM) protein (*N* x 4.67), 11.9% (DM) EE, 7.65% (DM) CA, and 14.6% (DM) CF (Marschall et al., 2023). The sugar and starch contents of the diets were quantified with the official methods (VDLUFA, 2012). The fatty acid composition of the diets was analyzed as described below. The apparent *N*-corrected metabolizable energy (**AME$_N$**) content of the diets was calculated following the formula in the Commission Directive 86/174/EEC (Commission of the European Communities 1986):

$$\begin{aligned} \mathrm{AME}_N(\mathrm{Mj/kg}) = [&(0.1551 * \% \text{ crude protein}) \\ &+ (0.3431 * \% \text{ ether extract}) \\ &+ (0.1669 * \% \text{ starch}) + (0.1301 * \% \text{ sugar})] \end{aligned}$$

## Sample Collection

For determination of carcass yield and breast muscle weights, all animals (4 per cage) were electrically anaesthetized using a BTG-40A stunning device (Westerhoff Geflügeltechnik, Hoogstede, Germany) and then killed by bleeding through simultaneously opening of *Vena jugularis* and *Arteria carotis* in accordance with the European legislation for euthanasia of animals (European Union, 2019). For the metabolic parameters, only 2 animals per cage, whose BW were the closest to the mean BW of the whole group, were used in order to avoid a possible bias by random selection of broilers with very low or very high body weights. The venous and arterial whole blood mixture was collected into ethylenediaminetetraacetic acid-coated polyethylene tubes (9 mL S-Monovette, Sarstedt, Numbrecht, Germany) and the plasma was separated from the whole blood by centrifugation (1,100 x g, 10 min) at 4°C, slowly frozen at −20°C and stored at −80°C. The liver was removed, washed with cold NaCl solution (0.9%), weighed, and several small aliquots were taken. The right and left breast muscle was excised, weighed, and small aliquots were taken from the right breast muscle. The gastrointestinal tract was removed and digesta from ileum (segment between Meckel's diverticulum and the ileo-cecal junction) and cecum were collected. Tissue and digesta samples were snap frozen in liquid nitrogen and stored at −80°C. The remainders of the right breast muscles were frozen at −20°C.

## Determination of AID Coefficients

The AID coefficients of amino acids were determined in the ileum chyme using $TiO_2$ as an indicator. The ileum digesta was freeze dried and manually grounded under $N_2$ with a mortar. The concentration of $TiO_2$ was determined in the chyme and the finisher diets following the method of Brandt and Allam (1987) with slight modifications. The determination of amino acids was carried out by official methods (VDLUFA, 2012). The following formula was used to calculate the AID of the different nutrients:

$$AID\ coefficient\ [\%] = 100 - \left[\left(\frac{TiO2\_Diet}{TiO2\_Chyme}\right) * \left(\frac{Nutrient\_Chyme}{Nutrient\_Diet}\right) * 100\right]$$

In which $TiO_{2\_Diet}$ and $TiO_{2\_Chyme}$ is the $TiO_2$ concentration (% DM) in the diet and the chyme, respectively, and $Nutrient_{\_Chyme}$ and $Nutrient_{\_Diet}$ is the nutrient concentration (% DM) in the chyme and the diet, respectively.

## Determination of Total Lipid Fatty Acid Composition of Diets and Liver

The total lipid fatty acid composition in diets and liver were analyzed by gas chromatography (**GC**). Prior to analysis, 100 mg diet and 150 mg liver, respectively, were extracted with 3 mL of 3:2 (v:v) n-hexane and isopropanol containing 50 mg/mL of C19:0 as internal standard for 18 h (Hara and Radin, 1978). The samples were centrifuged at 1,200 x g at room temperature for 15 min and the supernatant was transferred into a fresh tube. A 500 $\mu$L-aliquot of the extract was evaporated with a $N_2$ stream at 30°C. The dried sample was solved in 1 mL methanolic sodium hydroxide, sonicated for 5 min, and incubated for 5 min at 90°C. 1 mL of $BF_3$ solution was added as methylation agent to the cooled samples and mixed by vortexing. The sample was incubated at 90°C for 30 min and afterwards extracted 2 times with 1 mL n-hexane for 5 min. The n-hexane fractions were combined, evaporated and the residue was solved in 70 $\mu$L n-hexane. The fatty acid methyl esters (**FAME**) were separated with a Clarus 580 GC system (Perkin Elmer, Waltham, MA) equipped with a polar capillary column (30 m, 0.25 m internal diameter, 0.25 $\mu$m film thickness; Phenomenex Ltd., Aschaffenburg, Deutschland, Germany) and a flame ionization detector. The injection volume was 1 $\mu$L with a 1:30 split and the carrier gas was helium with a flow rate of 3.9 mL/min at the beginning. The following temperature program was used: 2 min at 120°C, then the temperature was increased at a rate of 2°C per min until 200°C was reached and held at 200°C for 5 min. A commercial standard mixture was used to identify the FAME by comparing the retention times of the standards with those of the sample.

## Determination of Short-Chain Fatty Acid (SCFA) Concentrations in the Cecal Digesta

The concentration of SCFA in cecal digesta was determined as described previously (Fiesel et al., 2014) using a Clarus 580 GC system (Perkin Elmer) equipped with a polar capillary column (10 m free fatty acid phase, 0.32 mm internal diameter, 0.25 $\mu$m film thickness; Macherey and Nagel, Düren, Germany) and a flame ionization detector. The concentrations of SCFA were calculated as $\mu$mol/g cecal digesta.

## Determination of Microbiota Composition and Diversity in the Cecal Digesta

For microbiota analysis, cecal digesta samples were sent on dry ice to Life and Brain GmbH (Bonn, Germany). Extraction of total genomic DNA from 120 mg-aliquots was performed using ZR BashingBead Lysis Tubes (0.1 and 0.5 mm, Zymo Research, Freiburg, Germany) and the Chemagic DNA Stool Kit (Perkin Elmer, Rodgau, Germany) according to the manufacturer's protocol. Amplicons were produced in a 2-step PCR reaction. Amplification of the V3/V4 region of the 16S rRNA gene was carried out in the first step using 2x KAPA HiFi HotStart ReadyMix (Roche, Mannheim, Germany) and the primers Bakt_341F (5′-CCTACGGGNGGCWGCAG-3′) and Bakt_805R (5′-GACTACHVGGGTATCT AATCC-3′) with the following PCR protocol: 3 min denaturation at 95°C and 25 cycles of denaturation (30 s at 95°C), annealing (30 s at 55°C), extension (30 s at 72°C), and final extension at 72°C for 5 min. The samples were spot-checked on an Agilent TapeStation 4200 using D1000 and purified with AMPure XP beads (Beckman Coulter, Krefeld, Germany). In the second PCR, dual indices and Illumina sequencing adapters were added to the amplicon ends in preparation for sequencing using the Nextera XT v2 Index Kit (Illumina, San Diego, CA). The reaction solution for the second PCR consisted of 25 $\mu$L of 2x KAPA HiFi HotStart ReadyMix (Roche, Mannheim, Germany), 5 $\mu$L of the corresponding Nextera XT index primer, 10 $\mu$L of PCR grade water, and 5 $\mu$L of purified product from the first PCR. The samples were subjected to the following PCR protocol: 3 min denaturation at 95°C and 25 cycles of denaturation (30 s at 95°C), annealing (30 s at 55°C), extension (30 s at 72°C), and final extension at 72°C for 5 min. The samples were spot-checked and purified like before. The concentrations of the samples were normalized to 4 nM and a pooled sample was prepared. The pooled sample was quantified using the Qubit dsDNA HS Assay Kit from Thermo Fisher Scientific (Waltham, MA), and fragment size examined on a D1000 ScreenTape. An Illumina MiSeq system was used to perform the sequencing with MiSeq Reagent Kit v3 for 2 × 300 cycles. Clustering was performed at 8 pM with a 30% spike-in of PhiX. Afterwards the MiSeq system was used for the demultiplexing. QIIME 2 version 2022.8 was used to process the 16S sequencing data (Bolyen et al., 2019). Raw sequencing data were deposited as FASTQ files at the NCBI's sequence read archive (**SRA**) repository and is available under bioproject ID PRJNA1079797. DADA2 was used for sequencing quality control, including filtering for PhiX reads and chimeric sequences, and denoising (Callahan et al., 2016). Afterwards, a pretrained SILVA classifier (silva-138-nr99-16S-V3-V4-classifier) was used to identify amplicon sequencing variants (**ASV**) with >99% similarity. The data was analyzed with the default settings of the MicrobiomeAnalyst platform for $\alpha$-diversity, $\beta$-diversity, and taxonomic analysis (Dhariwal et al., 2017; Chong et al., 2020).

## Total RNA Extraction and Hepatic Transcript Profiling

Total RNA was isolated from approximately 25 mg liver tissue with TRIzol reagent according to the

manufacturer's protocol (Invitrogen, Karlsruhe, Germany). RNA quantity and quality were determined spectrophotometrically with a 200M microplate reader equipped with a NanoQuant plate (Tecan, Mainz, Germany). The average RNA concentration and the A260/A280 ratio of all samples was 922 ± 252 ng/$\mu$L and 1.92 ± 0.02 ($N$ = 36, mean ± SD), respectively. Six RNA samples per group were randomly selected and sent on dry ice to the Genomics Core Facility "KFB - Center of Excellence for Fluorescent Bioanalytics" (Regensburg, Germany). Prior to processing, RNA quality was tested using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). An average RNA integrity number (RIN) value of 8.09 ± 0.32 ($N$ = 18, mean ± SD) indicated sufficient RNA quality to perform microarray analysis by Affymetrix GeneChip Array "Chicken Gene 1.0 ST", which covers 18,214 genes represented by 439,582 probes (Applied Biosystems GeneChip Whole Transcript (**WT**) PLUS Reagent Kit User Guide; Thermo Fisher Scientific). The processed GeneChips were scanned and cell intensity files were created based on the image data using the Command Console software (Affymetrix). Compressed array image files (CEL files) were imported into the Applied Biosystems Transcriptome Analysis Console (v. 4.0.2) software (Thermo Fisher Scientific) for the calculation of summarized probe set signals (in log2 scale) using the Robust Multichip Analysis algorithm, comparison fold changes (**FC**) and significance $P$-values (ANOVA). Gene names were assigned with the "ChiGene-1_0-st-v1.na36.galgal3.transcript.csv" annotation file. The microarray data of this study have been uploaded in MIAME compliant format in the NCBI's Gene Expression Omnibus (**GEO**) public repository and is available under GEO accession number GSE255945. Adjustment for false discovery rate (**FDR**) was not performed, because all adjusted $P$-values were > 0.05. Transcripts were classified as differentially expressed when the FC was > 1.3 or < -1.3 and the $P$-value was < 0.05. Similar cut-off values were used in recent studies for the selection of differentially expressed genes (Gessner et al., 2019; Ringseis et al., 2019; Schäfer et al., 2023b). In order to identify enriched Gene Ontology (**GO**) terms the differentially expressed transcripts were uploaded into the Database for Annotation, Visualization, and Integrated Discovery 6.8 bioinformatic resource to perform a gene set enrichment analysis (**GSEA**) (Da Huang et al., 2009). GO terms were considered as enriched at $P$-value < 0.05.

## qPCR Analysis

qPCR analysis was carried out for validation of microarray data (16 genes) and for determination of the hepatic expression of genes involved in lipid synthesis (7 genes). The synthesis of the cDNA was performed as described previously (Chiappisi et al., 2017). Gene specific primer pairs were designed with Primer3 and synthesized by Eurofins MWG Operon (Ebersberg, Germany). Characteristics of primer pairs are listed in Supplemental Table 1. qPCR analysis was performed with a Rotor-Gene Q system (Qiagen, Hilden, Germany) as described earlier (Keller et al., 2012). Normalization was performed using the GeNorm procedure according to Vandesompele et al. (2002).

## Determination of Triglyceride (TG) and Cholesterol Concentrations in Liver and Plasma

The concentrations of TG and cholesterol were determined in plasma and lipid extracts of liver tissue with commercial enzymatic kits (Cholesterol: Fluitest CHOL, DiaSys Diagnostic Systems; TG: Fluitest TG, Analyticon Biotechnologies). Lipid extracts were prepared by homogenization of 70 mg liver aliquots with 1.5 mL of 3:2 (v:v) n-hexane and isopropanol and subsequent incubation for 18 h at room temperature. Prior to measurement, aliquots of the lipid extracts were evaporated under a constant $N_2$ flow at 37°C, dissolved in 25 $\mu$L Triton X-100 chloroform (1:1, v:v) and incubated overnight (Eder and Kirchgessner, 1994).

## Hepatic Activities of the Lipogenic Enzyme Malic Enzyme

The activity of malic enzyme (**ME**, EC 1.1.1.40) was determined in the cytosolic fraction of homogenized liver tissue. Cytosolic fractions were prepared according to Maheshwari et al. with slight modifications in the amount of sample (100 mg) and buffer (1.5 mL) used (Maheshwari et al., 2020). The assay was started by adding malate to a mixture of cytosolic fraction and buffer containing $NADP^+$. ME activity was determined spectrophotometrically by measuring the decrease of $NADP^+$. The activity was normalized by the total protein concentration of the cytosolic fraction (Geer et al., 1979).

## Targeted Metabolite Screening

Quantification of targeted plasma metabolites was carried out with a combination of liquid chromatography (Agilent 1290 Infinity II LC, Santa Clara, CA) and mass spectrometry (SCIEX 5500 QTrap MS, Darmstadt, Germany) using the MxP Quant 500 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) as described recently in more detail (Marschall et al., 2023). The metabolomics dataset was subjected to principal component analysis (**PCA**). Prior to the PCA, variables with missing values were either excluded from the analyzes if more than 50 % of the samples were missing or the missing values were replaced by the limit of detection. After normalization by log-transformation and autoscaling the remaining values were used for the PCA.

**Table 2.** Chemical composition of the broiler diets.

| | Starter | | | Grower | | | Finisher | | |
|---|---|---|---|---|---|---|---|---|---|
| | HI0 | HI7.5 | HI15 | HI0 | HI7.5 | HI15 | HI0 | HI7.5 | HI15 |
| Analyzed concentration of crude nutrients and energy | | | | | | | | | |
| DM, % FM | 86.1 | 85.7 | 86.6 | 85.7 | 86.8 | 86.3 | 86.6 | 87.5 | 87.9 |
| $AME_N$, kcal/kg DM | 3,344 | 3,272 | 3,344 | 3,392 | 3,368 | 3,416 | 3,439 | 3,439 | 3,416 |
| GE, kcal/kg DM | 5,064 | 5,111 | 4,801 | 5,064 | 5,040 | 4,825 | 4,992 | 4,992 | 5,016 |
| $CP_{Chitin\text{-}corr}$[1], % DM | 23.3 | 24.2 | 24.5 | 21.4 | 21.8 | 22.1 | 20.1 | 21.2 | 21.2 |
| CF, % DM | 4.1 | 4.8 | 5.5 | 4.0 | 4.6 | 4.8 | 3.8 | 4.3 | 4.7 |
| EE, % DM | 9.9 | 9.4 | 9.6 | 9.8 | 9.5 | 9.3 | 9.4 | 9.4 | 9.7 |
| CA, % DM | 6.6 | 6.7 | 7.1 | 6.1 | 6.3 | 6.3 | 6.3 | 6.4 | 6.5 |
| Starch, % DM | 39.4 | 38.3 | 38.6 | 42.8 | 42.9 | 43.8 | 45.8 | 44.7 | 44.5 |
| Sugar, % DM | 3.4 | 2.7 | 3.4 | 3.1 | 2.4 | 2.9 | 3.0 | 3.3 | 2.0 |
| Digestible amino acids, g/kg DM[2] | | | | | | | | | |
| Lysine | 12.93 | 13.93 | 13.93 | 12.33 | 12.08 | 12.01 | 10.93 | 10.8 | 10.68 |
| Methionine | 6.46 | 6.90 | 7.06 | 6.13 | 6.34 | 6.41 | 5.36 | 5.88 | 6.06 |
| Cysteine | 3.34 | 3.18 | 3.01 | 3.26 | 2.88 | 2.68 | 3.12 | 2.80 | 2.59 |
| Methionine + Cysteine | 9.79 | 10.08 | 10.07 | 9.38 | 9.22 | 9.09 | 8.48 | 8.68 | 8.65 |
| Tryptophan | 2.33 | 2.61 | 2.76 | 2.31 | 2.27 | 2.41 | 2.07 | 2.29 | 2.28 |
| Threonine | 8.74 | 9.04 | 9.15 | 8.03 | 8.08 | 7.90 | 7.17 | 7.23 | 7.11 |
| Arginine | 13.26 | 13.24 | 12.80 | 12.76 | 11.94 | 11.42 | 11.40 | 10.79 | 10.29 |
| Valine | 10.14 | 10.21 | 10.17 | 9.62 | 9.59 | 9.37 | 8.49 | 8.43 | 8.90 |
| Isoleucine | 8.57 | 8.83 | 8.92 | 8.29 | 8.09 | 8.00 | 7.57 | 7.44 | 7.43 |
| Fatty acids[3], g/100 g of total fatty acids | | | | | | | | | |
| C12:0 | <0.1 | 4.45 | 9.14 | <0.1 | 4.30 | 9.33 | <0.1 | 4.16 | 9.70 |
| C14:0 | 0.08 | 0.97 | 1.87 | 0.07 | 0.92 | 1.93 | 0.08 | 0.91 | 2.03 |
| C16:0 | 12.3 | 12.4 | 12.6 | 12.6 | 12.4 | 12.6 | 12.2 | 12.3 | 12.5 |
| C16:1 | 0.11 | 0.38 | 0.69 | 0.10 | 0.37 | 0.71 | 0.10 | 0.36 | 0.74 |
| C18:0 | 3.56 | 3.35 | 3.11 | 3.75 | 3.27 | 3.06 | 3.43 | 3.21 | 2.98 |
| C18:1 n-9 | 27.7 | 26.0 | 24.3 | 27.7 | 26.2 | 24.4 | 27.6 | 26.0 | 24.2 |
| C18:2 n-6 | 51.0 | 47.5 | 43.7 | 50.7 | 47.8 | 43.6 | 51.4 | 48.2 | 43.5 |
| C18:3 n-3 | 4.4 | 4.0 | 3.7 | 4.2 | 4.0 | 3.6 | 4.4 | 4.1 | 3.6 |

[1]$CP_{Chitin\text{-}corr}$, % DM [(total *N* - Chitin *N*) x 6.25]

[2]Calculation was based on the digestibility reported in Schiavone et al. (2017) for *Hermetia illucens* larvae meal, and Sauvant et al. (2004) for the other components.

[3]Only fatty acids with a content above 0.5 % in at least one diet are listed.Abbreviations: $AME_N$, Apparent *N*-corrected metabolizable energy; CA, crude ash; CF, crude fiber; DM, dry matter; EE, ether extract; FM, fresh matter; GE, gross energy.

### Statistical Analysis

The statistical analyses were carried out using SPSS 28 software (IBM, Armonk, NY). The experimental unit was the cage for feed intake and feed: gain ratio and the individual animal for all other data. Normal distribution was tested using the Shapiro-Wilk test and homoscedasticity was evaluated using the Levenés test. Normally distributed data with homogeneous variances were analyzed by one-way ANOVA followed by Tukeýs post-hoc test, data with heterogeneous variances were analyzed by Welchś ANOVA in combination with the Games-Howell post-hoc test. Data that were not normally distributed were log-transformed and re-tested for normal distribution. Log-normally distributed data were analyzed as before. If the data were not normally distributed, the non-parametric Kruskal-Wallis test was performed, followed by the Mann-Whitney U test with Bonferroni correction. For all data except metabolomics data, differences with $P < 0.05$ were considered significant. The metabolomics data was log-transformed and subjected to one-way ANOVA with Tukey's post-hoc test. Metabolites with an FDR-adjusted *P*-value < 0.05 were considered significantly different.

## RESULTS

### Composition of the Experimental Diets

The composition of the 3 experimental diets within a phase was similar regarding the concentrations of DM, EE, starch, sugar, gross energy, and $AME_N$ (Table 2). The concentration of CF within a phase was increased with increasing inclusion level of HI larvae meal due to the presence of chitin. The concentration of chitin-corrected protein was similar between the diets containing HI larvae meal within a phase but was slightly lower in diet HI0. The digestible amounts of the essential amino acids were comparable across the 3 diets within a phase, with the exception of lysine in the starter diets; the digestible amount of lysine was 1 g/kg diet lower in the HI0 diet than in the diets HI7.5 and HI15.

As expected, the fatty acid profile of dietary total lipids differed depending on the inclusion level of HI larvae meal. In all diets, C18:2 n-6, C18:1 n-9 and C16:0 were the dominating fatty acids followed by C18:3 n-3 in the HI0 diets and C12:0 in the 2 diets containing HI larvae meal (HI7.5 and HI15). The proportions of C12, C14, and C16:1 in the dietary total lipids increased in the order HI0 < HI7.5 < HI15 within each phase, whereas those of C18:0, C18:1 n-9, C18:2 n-6 and C18:3 n-3 decreased in the order HI15 < HI7.5 < HI0 within each phase.

**Table 3.** Growth and slaughter performance data of broilers fed diets with either 0% (HI0), 7.5% (HI7.5) or 15% (HI15) HI larvae meal for 35 d.

| | HI0 | HI7.5 | HI15 | *P*-value |
|---|---|---|---|---|
| Growth performance | | | | |
| Whole period (d 1−35) | | | | |
| Initial BW, g | 40.9 ± 2.2 | 40.8 ± 2.3 | 40.8 ± 2.2 | 0.984 |
| Final BW, g | 2725 ± 233 | 2794 ± 274 | 2837 ± 217 | 0.268 |
| BW gain, g | 2684 ± 233 | 2753 ± 273 | 2796 ± 217 | 0.255 |
| Feed intake, g | 3795 ± 177 | 3874 ± 189 | 3869 ± 148 | 0.681 |
| Feed:gain ratio, g/g | 1.41 ± 0.04 | 1.41 ± 0.03 | 1.38 ± 0.05 | 0.307 |
| Starter phase (d 1−10) | | | | |
| BW gain, g | 274 ± 28 | 273 ± 32 | 284 ± 26 | 0.495 |
| Feed intake, g | 296 ± 19 | 292 ± 21 | 299 ± 15 | 0.821 |
| Feed:gain ratio, g/g | 1.09 ± 0.04 | 1.08 ± 0.06 | 1.05 ± 0.02 | 0.298 |
| Grower phase (d 11−21) | | | | |
| BW gain, g | 842 ± 71 | 859 ± 73 | 853 ± 58 | 0.698 |
| Feed intake, g | 1107 ± 67 | 1102 ± 61 | 1097 ± 33 | 0.953 |
| Feed:gain ratio, g/g | 1.31 ± 0.01 | 1.28 ± 0.05 | 1.28 ± 0.03 | 0.216 |
| Finisher phase (d 21−35) | | | | |
| BW gain, g | 1564 ± 226 | 1630 ± 227 | 1652 ± 177 | 0.368 |
| Feed intake, g | 2391 ± 169 | 2480 ± 129 | 2473 ± 118 | 0.497 |
| Feed:gain ratio, g/g | 1.53 ± 0.09 | 1.54 ± 0.04 | 1.49 ± 0.10 | 0.537 |
| Slaughter performance | | | | |
| Carcass yield, % | 72.0 ± 2.3[b] | 73.5 ± 2.7[ab] | 74.3 ± 2.8[a] | 0.012 |
| Breast muscle weight, g | 582 ± 71[b] | 639 ± 102[ab] | 661 ± 80[a] | 0.008 |

Values are means ± SD for $n = 24$ broilers/group (Initial BW, final BW, BW gain, carcass yield, breast muscle weight) and $n = 6$ cages/group (feed intake, feed:gain ratio).

[a,b]Means without a common letter differ across the groups, $P < 0.05$.Abbreviation: BW, Body weight.

## Growth Performance

Performance parameters were not different between the three groups during neither the individual phases nor the whole period (Table 3). Carcass yield and breast muscle weight were higher in group HI15 than in group HI0 ($P < 0.05$), whereas these parameters did not differ between group HI7.5 and the other 2 groups.

## AID of Amino Acids

Amongst the amino acids analyzed, the AID of 13 amino acids differed between the groups ($P < 0.05$). The AID of methionine, phenylalanine and serine was higher in group HI15 than in groups HI7.5 and HI0, while that of tyrosine and threonine was higher only in group HI15 than in group HI0 ($P < 0.05$). The AID of arginine, asparagine, glutamine, glycine, leucine, isoleucine and valine was higher in group HI15 than in group HI7.5 ($P < 0.05$) but was not different between group HI15 and group HI0 (Table 4). The AID of alanine, cysteine, histidine, lysine, and proline did not differ between groups.

**Table 4.** AID coefficients for amino acids of broilers fed diets with either 0% (HI0), 7.5% (HI7.5) or 15% (HI15) HI larvae meal for 35 d.

| AID, % | HI0 | HI7.5 | HI15 | *P*-value |
|---|---|---|---|---|
| Indispensable amino acids | | | | |
| Arginine | 79.3 ± 7.3[ab] | 80.4 ± 4.6[b] | 84.5 ± 2.5[a] | 0.011 |
| Histidine | 73.8 ± 8.0 | 75.2 ± 4.9 | 79.0 ± 3.6 | 0.059 |
| Leucine | 73.7 ± 8.7[ab] | 76.3 ± 5.4[b] | 81.2 ± 3.3[a] | 0.008 |
| Lysine | 78.6 ± 6.9 | 79.3 ± 4.4 | 83.7 ± 3.8 | 0.069 |
| Isoleucine | 71.3 ± 9.2[ab] | 73.3 ± 5.6[b] | 78.8 ± 4.1[a] | 0.014 |
| Methionine | 86.3 ± 4.8[b] | 88.7 ± 3.2[b] | 91.5 ± 2.8[a] | 0.009 |
| Phenylalanine | 74.2 ± 8.8[b] | 76.7 ± 5.5[b] | 82.4 ± 3.8[a] | 0.006 |
| Threonine | 65.7 ± 9.1[b] | 68.7 ± 6.4[ab] | 74.9 ± 5.5[a] | 0.022 |
| Valine | 71.9 ± 8.5[ab] | 72.7 ± 5.7[b] | 78.0 ± 4.2[a] | 0.031 |
| Dispensable amino acids | | | | |
| Alanine | 61.0 ± 7.9 | 59.5 ± 8.6 | 63.2 ± 5.7 | 0.421 |
| Asparagine | 73.6 ± 7.6[ab] | 74.6 ± 5.4[b] | 79.8 ± 3.0[a] | 0.008 |
| Cysteine | 64.3 ± 9.4 | 65.0 ± 8.1 | 69.4 ± 5.2 | 0.153 |
| Glutamine | 79.4 ± 7.3[ab] | 80.6 ± 4.6[b] | 84.8 ± 2.4[a] | 0.009 |
| Glycine | 67.2 ± 8.2[ab] | 68.3 ± 5.4[b] | 73.2 ± 3.8[a] | 0.022 |
| Proline | 74.2 ± 9.2 | 75.9 ± 6.1 | 80.6 ± 3.6 | 0.034 |
| Serine | 69.7 ± 8.8[b] | 71.9 ± 5.7[b] | 77.1 ± 4.0[a] | 0.020 |
| Tyrosine | 72.7 ± 9.0[b] | 76.3 ± 5.4[ab] | 81.0 ± 4.0[a] | 0.013 |

Values are means ± SD for $n = 12$ broilers/group.

[a,b]Means without a common letter differ across the groups, $P < 0.05$.
Abbreviation: AID, apparent ileal digestibility.

## Microbiota Diversity and Composition in the Cecal Digesta

Following data normalization and filtering, a total of 77 ASV were used for the analysis of microbiota diversity and composition. Four indicators (Chao1, Observed, Shannon index, Simpson index) were used to examine the $\alpha$-diversity (Figure 1A). Chao1 and Observed, both of which are indicators of the richness of the microbial community, were higher in groups HI7.5 and HI15 than in group HI0 ($P < 0.05$). The Shannon index, which considers both the richness and the evenness, tended to be higher in groups HI7.5 and HI15 than in group HI0 ($P = 0.059$), whereas no effect was seen for the Simpson index ($P = 0.582$). For the assessment of the $\beta$-diversity, 3 metrics (Bray-Curtis index, Jensen-Shannon divergence, Jaccard index) were calculated and the data were visualized as NMDS plots (Figure 1B). While the Jaccard index ($P = 0.038$) and the Bray-Curtis index ($P = 0.058$) showed or tended to show a difference between groups, the Jensen-Shannon divergence ($P = 0.177$) did not differ between groups.

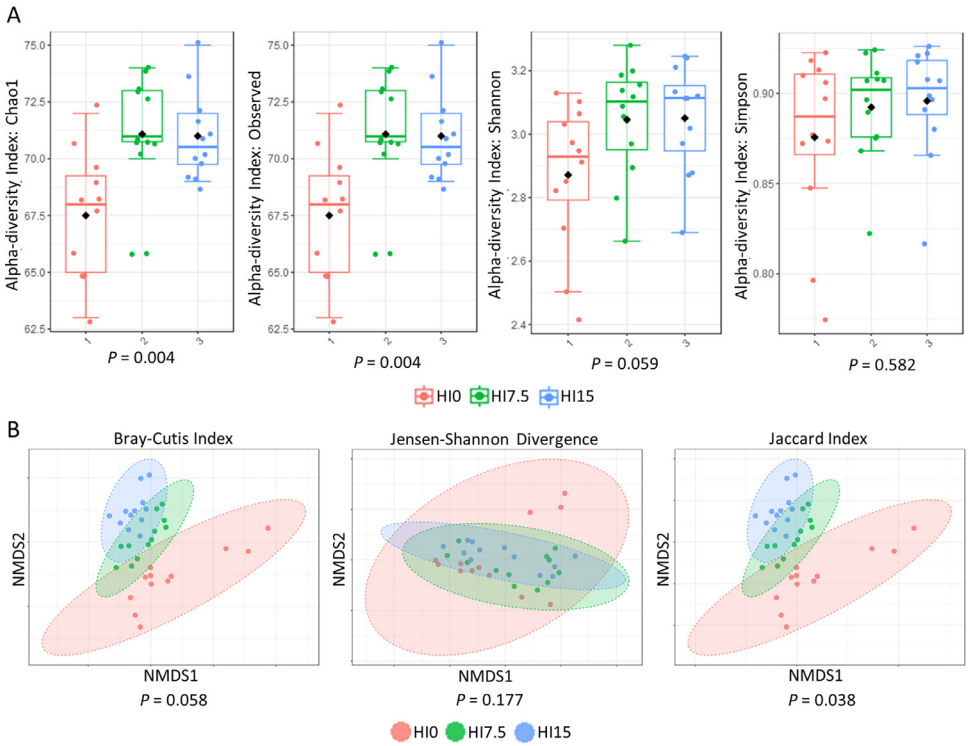


**Figure 1.** Analysis of the microbiota diversity in the cecal digesta. (A) $\alpha$-diversity indicators (Chao1, observed, Shannon and Simpson) and (B) $\beta$-diversity metrics (Bray-Curtis Index, Jensen-Shannon Divergence, Jaccard Index) of the cecal bacterial community. $\alpha$-diversity indicators are shown as box plots and $\beta$-diversity metrics as NMDS plots for $n = 12$ broilers/group.

The effect on the microbiota composition was analyzed at different taxonomic levels (phylum, class, order, family, genus; Figure 2). Analysis at the phylum level showed that the cecum digesta samples contained bacteria from only three different phyla (Firmicutes, Proteobacteria, Verrumicrobiota), whose abundance did not differ across the 3 groups. The by far most dominant phylum was Firmicutes (> 97% in all groups), followed by Proteobacteria (1.4−2.2% in all groups) and Verrumicrobiota (0.1−0.3% in all groups). At the order level, 14 orders could be identified, amongst which Lachnospirales (53−58% in all groups) and Lactobacillales (15−22%) were the dominant taxa in the cecal digesta. Differences in the abundance between groups at the order level were found only for two of the low-abundant orders; that is, the abundance of Paenibacillales and Clostridia vadinBB60 group was higher in groups HI7.5 and HI15 than in group HI0 ($P < 0.05$, Table 5). At the family level, 24 families were detected and the abundance of 5 families, all of which belonged to the phylum Firmicutes, differed between the groups (Table 5). The abundance of Paenibacillaceae, Ruminococcaceae, Clostridia vadinBB60 group and an uncultured family of Oscillospirales in the cecal digesta was higher in groups HI7.5 and HI15 than in group HI0 ($P < 0.05$), whereas that of Butyricicoccaceae was lower in group HI15 than in groups HI0 and HI7.5 ($P < 0.05$). At the genus level, 53 genera could be identified, amongst which *Lactobacillus* and an unknown genus from Lachnospiraceae were the dominating taxa with 19 to 22% and 19 to 24% respectively, in the cecal digesta. The abundance of 18 genera was found to differ between the 3 groups (Table 5). The abundance of *Clostridia_vadinBB60_group*, *Lachnoclostridium*, *DTU089* (Ruminococcaceae), *Paenibacillus, Paludicola,* and a genus belonging to *Ruminococcaceae incertae sedis* was higher in groups HI7.5 and HI15 than in group HI0 ($P < 0.05$). The abundance of *Shuttleworthia* and 2 uncultured genera from *Oscillospirales* and *Oscillospiraceae*, respectively, was lower in group HI15 than in groups HI0 and HI7.5 ($P < 0.05$). The abundance of *Oscillibacter* was lower and that of *Butyricicoccus* and an unknown genus from *Peptostreptococcaceae* was higher in group HI15 than in groups HI0 and HI7.5 ($P < 0.05$). The abundance of *Caproiciproducens* was higher in group HI15 than in group HI7.5 and higher in group HI7.5 than in group HI0 ($P < 0.05$). The abundance of *GCA_900066575* from Lachnospiraceae and *Romboutsia* was lower and that of *Eubacterium hallii group* was higher in group HI7.5 than in group HI0.5 ($P < 0.05$). The abundance of *UCG-009* (Butyricicoccaceae) and an unknown genus from Oscillospiraceae was higher in group HI15 than in group HI7.5 ($P < 0.05$).

## Concentrations of SCFA in the Cecal Digesta

While the concentration of total SCFA in the cecal digesta did not differ among groups ($P = 0.282$, Figure 3A), the concentration of butyric acid was lower in group HI15 than in group HI0 ($P < 0.05$, Figure 3B) and the concentrations of isobutyric acid and valeric acid were lower in group HI15 than in group HI7.5 ($P < 0.05$, Figure 3C). The concentrations of acetic acid ($P = 0.787$), propionic acid ($P = 0.219$), and isovaleric acid ($P = 0.141$) in the cecal digesta did not differ among groups.

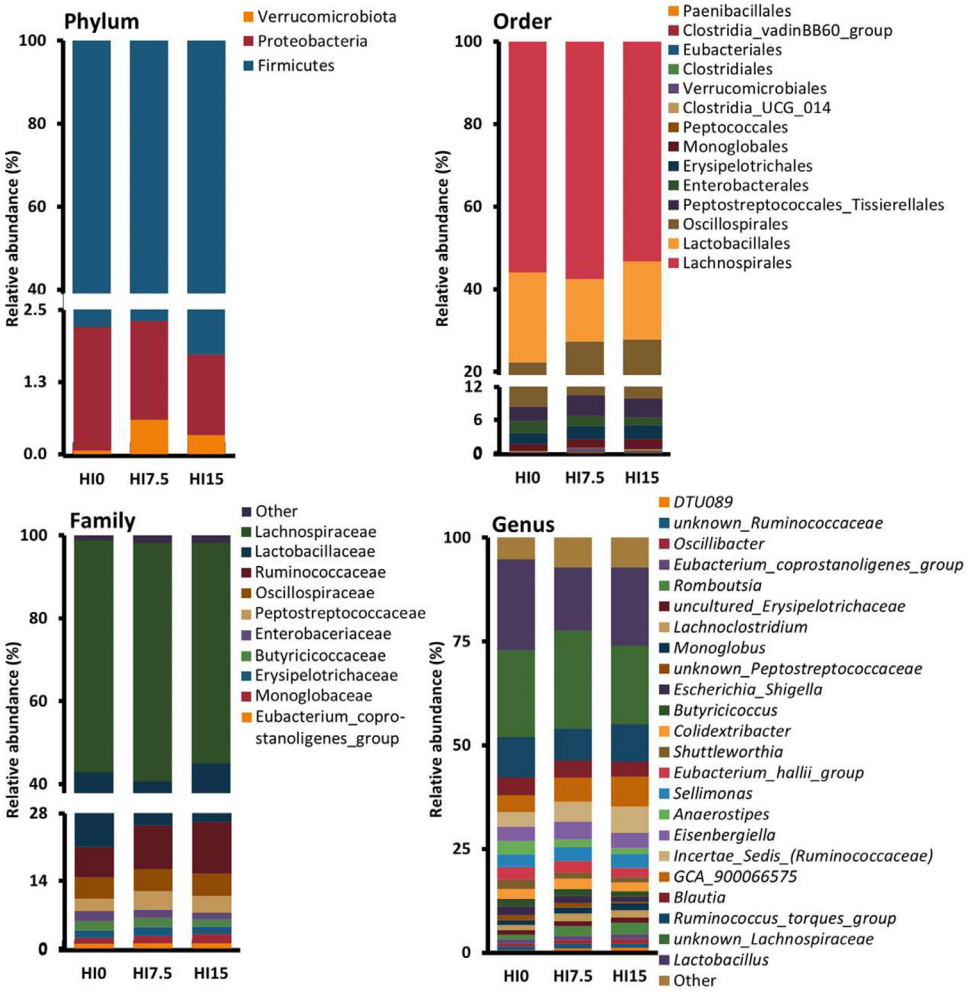


**Figure 2.** Distribution of cecal bacteria at different taxonomic levels (phylum, order, family, genus) of broilers fed diets with either 0% (HI0), 7.5% (HI7.5), or 15% (HI15) HI larvae meal for 35 d. Families and genera with less than 1% in all groups were summarized as other. Data are means for $n = 12$ broilers/group.

## Liver Transcriptome

Regarding the comparison of group HI15 vs. group HI0, 33 and 37 genes were found to be up- and down-regulated, respectively, according to the filter criteria applied (Supplemental Table 2). The ten most strongly up-regulated genes were (FC in brackets): *DDX60* (2.96), *ETNPPL* (2.45), *CACNA1D* (1.89), *LOC422154* (1.76), *CERS6* (1.74), *PCDH7* (1.64), *PAPLN* (1.64), *CYP1A4* (1.50), and *BHMT* (1.49). The down-regulation was weaker, with a maximum FC of -1.67 for *STARD4*. Besides STARD4, the following genes belonged to the ten most strongly down-regulated genes (FC in brackets): *TRIM2* (-1.55), *RAB38* (-1.54), *MIR34B* (-1.49), *LOC418189* (-1.45), *MIRLET7F2* (-1.45), *AACS* (-1.44), *HMGCR* (-1.44), *LOC101749997* (-1.42), and *MIR1671* (-1.42).

For the comparison of group HI7.5 vs. group HI0, 20 up-regulated and 41 down-regulated genes could be identified (Supplemental Table 3). The ten most strongly up-regulated genes were (FC in brackets): *MAP3K7Cl* (1.99) followed by *PCDH7* (1.67), *CREB5* (1.52), *SNORD20* (1.45), *ACPL2* (1.44), *MIR365-2* (1.43), *MICAL3* (1.42), *PAPLN* (1.39), *GUCY1A2* (1.39), and *MIR181A1* (1.36). *MIR34B* was the gene with the strongest down-regulation (FC -1.81) followed by (FC in brackets): *TRPM8* (-1.64), *MIRLET7F1* (-1.59), *MIR1556* (-1.54), *MIR1796* (-1.52), *ACKR4* (-1.51), *MIR1682* (-1.47), *GAL13* (-1.45), *HMMR* (-1.45), and *LRRC18* (-1.43).

Validation of microarray data was carried out by qPCR measurement of 16 strongly regulated genes. Comparing the FC from microarray and qPCR showed that the regulatory direction observed from microarray could be confirmed by qPCR for all genes, except *RAB38* (Table 6). However, only 4 genes were found to be regulated at $P < 0.05$ according to qPCR.

In the next step, GSEA was performed to identify biological processes affected by the differentially expressed genes. Regarding the comparison HI15 vs. HI0, GSEA showed two enriched biological process terms for the up-regulated genes and 13 for the down-regulated genes (Table 7). The following biological process terms were enriched within the list of up-regulated genes (sorted by enrichment *P*-value in increasing order): "de novo" IMP biosynthetic process, and regulation of heart rate by cardiac conduction. For the list of down-regulated genes the enriched biological process terms were (sorted by enrichment *P*-value in increasing order): transmitter-gated ion channel activity involved in regulation of

**Table 5.** Relative abundance of bacterial taxa found to be different in the cecum digesta of broilers fed diets with either 0% (HI0), 7.5% (HI7.5) or 15% (HI15) HI larvae meal for 35 d.

| | HI0 | HI7.5 | HI15 | FDR |
|---|---|---|---|---|
| Order | | | | |
| Clostridia_vadinBB60_group | 0.02 ± 0.01[b] | 0.17 ± 0.37[a] | 0.05 ± 0.03[a] | 0.009 |
| Paenibacillales | ND[b] | 0.02 ± 0.02[a] | 0.03 ± 0.01[a] | 0.000 |
| Family | | | | |
| Butyricicoccaceae | 1.95 ± 0.45[a] | 1.92 ± 0.40[a] | 1.48 ± 0.28[b] | 0.023 |
| Clostridia_vadinBB60_group | 0.02 ± 0.01[b] | 0.17 ± 0.37[a] | 0.05 ± 0.03[a] | 0.007 |
| Paenibacillaceae | n.d.[b] | 0.02 ± 0.02[a] | 0.03 ± 0.01[a] | 0.000 |
| Ruminococcaceae | 6.40 ± 1.60[b] | 9.09 ± 1.44[a] | 10.60 ± 2.30[a] | 0.001 |
| uncultured_Oscillospirales | >0.01[b] | 0.02 ± 0.01[a] | 0.04 ± 0.02[a] | 0.001 |
| Genera | | | | |
| *Butyricicoccus* | 1.80 ± 0.50[a] | 1.78 ± 0.39[a] | 1.27 ± 0.28[b] | 0.006 |
| *Caproiciproducens* | 0.09 ± 0.03[c] | 0.25 ± 0.09[b] | 0.42 ± 0.13[a] | 0.000 |
| *Clostridia_vadinBB60_group* | 0.02 ± 0.01[b] | 0.17 ± 0.37[a] | 0.05 ± 0.03[a] | 0.006 |
| *DTU089* | 0.64 ± 0.19[b] | 01.00 ± 0.21[a] | 1.09 ± 0.21[a] | 0.002 |
| *Eubacterium_hallii_group* | 2.96 ± 0.51[a] | 2.68 ± 0.72[ab] | 2.25 ± 0.33[b] | 0.027 |
| *GCA_900066575* | 4.12 ± 2.45[b] | 5.78 ± 2.59[ab] | 7.25 ± 2.39[a] | 0.037 |
| *Incertae_Sedis_(Ruminococcaceae)* | 3.55 ± 0.75[b] | 4.88 ± 1.00[a] | 6.23 ± 1.68[a] | 0.002 |
| *Lachnoclostridium* | 1.12 ± 0.44[b] | 1.89 ± 0.57[a] | 1.67 ± 0.52[a] | 0.015 |
| *Oscillibacter* | 0.67 ± 0.35[b] | 0.79 ± 0.17[b] | 1.04 ± 0.18[a] | 0.006 |
| *Paenibacillus* | n.d.[b] | 0.02 ± 0.02[a] | 0.03 ± 0.01[a] | 0.000 |
| *Paludicola* | 0.31 ± 0.16[b] | 0.53 ± 0.23[a] | 0.67 ± 0.23[a] | 0.006 |
| *Romboutsia* | 1.04 ± 1.27[b] | 2.27 ± 2.53[ab] | 2.76 ± 1.63[a] | 0.023 |
| *Shuttleworthia* | 2.24 ± 0.60[a] | 1.52 ± 0.37[b] | 1.23 ± 0.31[b] | 0.001 |
| *UCG_009* | 0.15 ± 0.12[ab] | 0.14 ± 0.03[b] | 0.22 ± 0.05[a] | 0.011 |
| *uncultured_Oscillospiraceae* | 0.32 ± 0.11[b] | 0.43 ± 0.09[a] | 0.54 ± 0.12[a] | 0.002 |
| *uncultured_Oscillospirales* | 0.01 ± 0.01[b] | 0.02 ± 0.01[a] | 0.04 ± 0.02[a] | 0.001 |
| *unknown_Oscillospiraceae* | 0.26 ± 0.16[ab] | 0.31 ± 0.07[b] | 0.43 ± 0.09[a] | 0.024 |
| *unknown_Peptostreptococcaceae* | 1.18 ± 1.28[a] | 0.98 ± 0.97[a] | 0.181 ± 0.24[b] | 0.006 |

Values are means ± SD for $n = 12$ broilers/group.
[a,b,c]Means without a common letter differ across the groups, $P < 0.05$.Abbreviation: n.d., not detected

postsynaptic membrane potential, postsynaptic membrane, inhibitory extracellular ligand-gated ion channel activity, sterol biosynthetic process, regulation of postsynaptic membrane potential, GABA-gated chloride ion channel activity, synaptic transmission; GABAergic, GABA-A receptor activity, cholesterol biosynthetic process, dendrite membrane, GABA-A receptor complex, gamma-aminobutyric acid signaling pathway, and chloride channel complex.

Interestingly, regarding the comparison HI7.5 vs. HI0, only one enriched biological process term (external side of plasma membrane) was identified, despite a comparable number of differentially regulated genes as for the comparison HI15 vs. HI0.

## TG and Cholesterol Concentrations in Liver and Plasma

Hepatic concentration of TG was higher in group HI15 than in group HI0 ($P < 0.05$). Hepatic concentration of cholesterol ($P = 0.695$) and plasma concentrations of TG ($P = 0.475$) and cholesterol ($P = 0.750$) did not differ among groups (Table 8).

## Hepatic Total Fatty Acid Concentration and Hepatic Total Lipid Fatty Acid Composition

Analysis of hepatic total lipid fatty acid composition revealed that the percentages of C12:0 and C14:0 were higher in the groups HI7.5 and HI15 than in group HI0, whereas the percentages of C18:0, C18:3n-6 C20:4 n-6 and C24:1 n-6 were lower in group HI15 than in group HI0 ($P < 0.05$, Table 9).

## Hepatic Expression and Activity of Enzymes Involved in Lipid Synthesis

The hepatic mRNA levels of *ACACA* ($P = 0.244$), *ACLY* ($P = 0.517$), *FASN* ($P = 0.259$), *LDLR* ($P = 0.790$), *SCD* ($P = 0.628$) and *HMGCR* ($P = 0.246$) did not differ among groups (Figure 4A). The mRNA level of *ME1* tended to be lower in group HI7.5 than in group HI0 ($P = 0.053$). Hepatic activity of ME showed no difference between groups ($P = 0.580$, Figure 4B).

## Targeted Plasma Metabolomics

A total of 514 metabolites out of 630 metabolites screened could be statistically evaluated. One hundred thirty-eight metabolites from 15 metabolite classes were different between the three groups (FDR < 0.05). These metabolites belonged predominantly to TG (91), glycerophospholipids (18), and amino acids related (8). The remaining metabolites belonged to amino acids (4), diacylglycerols (4), cholesterol esters (3), acylcarnitines (2), biogenic amines (1), ceramides (1), fatty acids (1), sphingolipids (1), alkaloids (1), amino oxides (1), carboxylic acids (1), and indoles derivates (1).

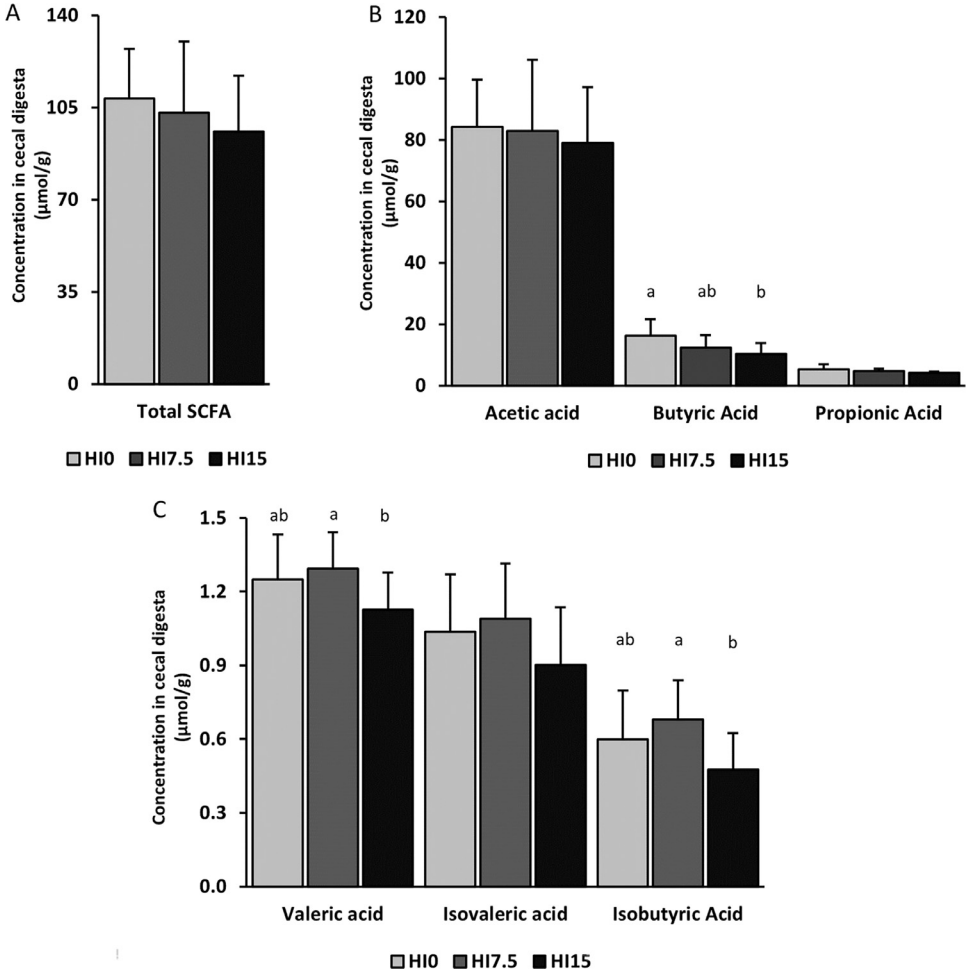


**Figure 3.** Concentrations of (A) total SCFA, (B) major SCFA (acetic acid, butyric acid, propionic acid), and (C) minor SCFA (valeric acid, isovaleric acid, isobutyric acid) in the cecal digesta of broilers fed diets with either 0% (HI0), 7.5% (HI7.5) or 15% (HI15) HI larvae meal for 35 d. Data are means ± SD for $n = 12$ broilers/group. [a,b]Means without a common letter differ across the groups, $P < 0.05$. Abbreviation: SCFA, short-chain fatty acids.

**Table 6.** qPCR validation of selected differentially regulated genes according to microarray for the comparison of groups HI15 vs. HI0.

| | Microarray | | qPCR | |
|---|---|---|---|---|
| | Fold change | *P*-value | Fold change | *P*-value |
| Up-regulated genes | | | | |
| *CERS6* | 1.74 | 0.008 | 1.51 | 0.148 |
| *PAPLN* | 1.64 | 0.023 | 2.27 | 0.075 |
| *CYP1A1* | 1.50 | 0.027 | 1.43 | 0.119 |
| *BHMT* | 1.49 | 0.045 | 2.90 | 0.001 |
| *ARRDC2* | 1.47 | 0.036 | 1.97 | 0.211 |
| *TDH* | 1.46 | 0.034 | 2.18 | 0.036 |
| *TFRC* | 1.43 | 0.010 | 1.27 | 0.242 |
| *PAICS* | 1.43 | 0.021 | 1.56 | 0.032 |
| Down-regulated genes | | | | |
| *STARD4* | −1.67 | 0.025 | −1.31 | 0.433 |
| *TRIM2* | −1.55 | 0.043 | −1.07 | 0.362 |
| *RAB38* | −1.54 | 0.018 | 1.08 | 0.770 |
| *AACS* | −1.44 | 0.025 | −1.54 | 0.028 |
| *HMGCR* | −1.44 | 0.014 | −1.19 | 0.504 |
| *AVD* | −1.41 | 0.014 | −2.60 | 0.093 |
| *PYGO* | −1.36 | 0.005 | −1.29 | 0.364 |
| *OTOG* | −1.36 | 0.001 | −1.29 | 0.468 |

Data are means for $n = 6$ (microarray) and $n = 12$ (qPCR).

The comparison between group HI15 and group HI0 showed the strongest difference of all comparisons considering that the concentrations of 134 metabolites differed. From the above listed metabolites only one TG (TG(17:1_38:7), one glycerophospholipid (lysoPC a C28:1), and one amino acid (alanine) were not different between group HI15 and group HI0.

Regarding the comparison between group HI7.5 and group HI0, the concentrations of 70 metabolites belonging to TG (46), glycerophospholipids (13), amino acids-related (4), amino acids (2), cholesterol esters (2), acylcarnitines (1), amino oxides (1), and indoles derivates (1) were found to be different.

Regarding the comparison between group HI15 and group HI7.5, the concentrations of 67 metabolites belonging to 12 metabolite classes were different. TG (44) were the dominant group followed by glycerophospholipids (7), amino acids-related (6), diacylglycerol (4), acylcarnitines (2), and one metabolite each from amino acids, cholesterol esters, amino oxides, biogenic amines, sphingolipids, alkaloids, and carboxylic acids. The list of all metabolites, whose concentrations differed among groups, can be found in Supplemental Table 4.

**Table 7.** Overview of the GO terms assigned to genes differentially regulated between groups HI15 vs. HI0 and groups HI7.5 vs. HI0.

| Term | Number of genes | *P*-value |
|---|---|---|
| HI15 vs. HI0: GO terms affected by up-regulated genes | | |
| de novo' IMP biosynthetic process | 2 | 0.012 |
| regulation of heart rate by cardiac conduction | 2 | 0.044 |
| HI15 vs. HI0: GO terms affected by down-regulated genes | | |
| sterol biosynthetic process | 2 | 0.015 |
| regulation of postsynaptic membrane potential | 2 | 0.015 |
| synaptic transmission, GABAergic | 2 | 0.018 |
| cholesterol biosynthetic process | 2 | 0.022 |
| gamma-aminobutyric acid signaling pathway | 2 | 0.026 |
| postsynaptic membrane | 3 | 0.007 |
| dendrite membrane | 2 | 0.022 |
| GABA-A receptor complex | 2 | 0.023 |
| chloride channel complex | 2 | 0.042 |
| transmitter-gated ion channel activity involved in regulation of postsynaptic membrane potential | 3 | 0.001 |
| inhibitory extracellular ligand-gated ion channel activity | 2 | 0.013 |
| GABA-gated chloride ion channel activity | 2 | 0.016 |
| GABA-A receptor activity | 2 | 0.021 |
| HI7.5 vs. HI0: GO terms affected by down-regulated genes | | |
| external side of plasma membrane | 3 | 0.043 |

**Table 8.** Concentrations of TG and cholesterol in plasma and liver of broilers fed diets with either 0% (HI0), 7.5% (HI7.5) or 15% (HI15) HI larvae meal for 35 d.

| | HI0 | HI7.5 | HI15 | *P*-value |
|---|---|---|---|---|
| Plasma, $\mu$mol/g | | | | |
| TG | 1.31 ± 0.37 | 1.26 ± 0.34 | 1.15 ± 0.25 | 0.475 |
| Cholesterol | 3.74 ± 0.44 | 3.67 ± 0.36 | 3.81 ± 0.47 | 0.750 |
| Liver, $\mu$mol/g | | | | |
| TG | 30.0 ± 5.5[b] | 35.0 ± 11.9[ab] | 38.4 ± 4.6[a] | 0.005 |
| Cholesterol | 12.7 ± 0.6 | 12.7 ± 1.4 | 12.3 ± 1.5 | 0.695 |

Data are means ± SD for $n = 12$ broilers/group.
[a,b]Means without a common letter differ across the groups, $P < 0.05$.Abbreviation: TG, triglycerides.

**Table 9.** Total lipid fatty acid composition and total fatty acid concentration in the liver of broilers fed diets with either 0% (HI0), 7.5% (HI7.5), or 15% (HI15) HI larvae meal for 35 d.

| | HI0 | HI7.5 | HI15 | *P*-value |
|---|---|---|---|---|
| Fatty acids, % of total fatty acids | | | | |
| C12:0 | 0.04 ± 0.01[a] | 0.12 ± 0.02[b] | 0.21 ± 0.06[c] | <0.001 |
| C14:0 | 0.33 ± 0.06[a] | 0.57 ± 0.12[b] | 0.88 ± 0.22[c] | <0.001 |
| C16:0 | 21.5 ± 3.4 | 22.0 ± 2.6 | 23.5 ± 2.4 | 0.232 |
| C16:1 n-7 | 1.34 ± 0.75 | 1.64 ± 0.87 | 1.91 ± 0.64 | 0.092 |
| C17:0 | 0.23 ± 0.05 | 0.21 ± 0.04 | 0.21 ± 0.04 | 0.655 |
| C18:0 | 22.3 ± 2.4[a] | 20.8 ± 2.2[ab] | 19.9 ± 2.0[b] | 0.041 |
| C18:1 n-9 | 18.5 ± 4.6 | 20.9 ± 4.0 | 22.6 ± 3.8 | 0.084 |
| C18:2 n-6 | 19.8 ± 2.5 | 20.2 ± 2.4 | 18.7 ± 3.1 | 0.423 |
| C18:3 n-6 | 0.15 ± 0.04[a] | 0.11 ± 0.02[b] | 0.11 ± 0.03[b] | 0.030 |
| C18:3 n-3 | 0.52 ± 0.09 | 0.53 ± 0.07 | 0.48 ± 0.16 | 0.070 |
| C20:0 | 0.14 ± 0.02 | 0.15 ± 0.03 | 0.13 ± 0.01 | 0.142 |
| C20:1n-9 | 0.32 ± 0.06[a] | 0.39 ± 0.06[b] | 0.35 ± 0.06[ab] | 0.019 |
| C20:2 n-6 | 0.68 ± 0.23 | 0.68 ± 0.30 | 0.62 ± 0.22 | 0.560 |
| C21:0 | 0.40 ± 0.11[a] | 0.59 ± 0.14[b] | 0.47 ± 0.13[ab] | 0.006 |
| C20:3 n-6 | 1.18 ± 0.20 | 1.04 ± 0.19 | 0.99 ± 0.21 | 0.080 |
| C20:4 n-6 | 10.1 ± 2.6[a] | 8.36 ± 2.02[ab] | 7.17 ± 1.57[b] | 0.011 |
| C22:0 | 0.08 ± 0.01 | 0.08 ± 0.02 | 0.07 ± 0.01 | 0.131 |
| C20:5 n-3 | 0.28 ± 0.07 | 0.23 ± 0.06 | 0.22 ± 0.06 | 0.069 |
| C24:1 n-9 | 0.76 ± 0.27[a] | 0.54 ± 0.17[ab] | 0.50 ± 0.16[b] | 0.017 |
| C22:6 n-3 | 1.42 ± 0.66 | 1.10 ± 0.33 | 1.04 ± 0.34 | 0.135 |

Data are means ± SD for $n = 12$ broilers/group.
[a,b]Means without a common letter differ across the groups, $P < 0.05$.

Two-dimensional reduction of the plasma metabolomes by PCA conducted with all plasma metabolites analyzed showed almost no overlap of the plasma metabolomes of groups HI15 and HI0 confirming the above-described results, while there was more overlap of the plasma metabolome of group HI7.5 with that of group HI0 and group HI15 (Figure 5). The 2 dimensions accounted for 42.6% of the variance in the metabolomics data set with principal component one describing 23.9% and principal component two 18.7%. The loading plot

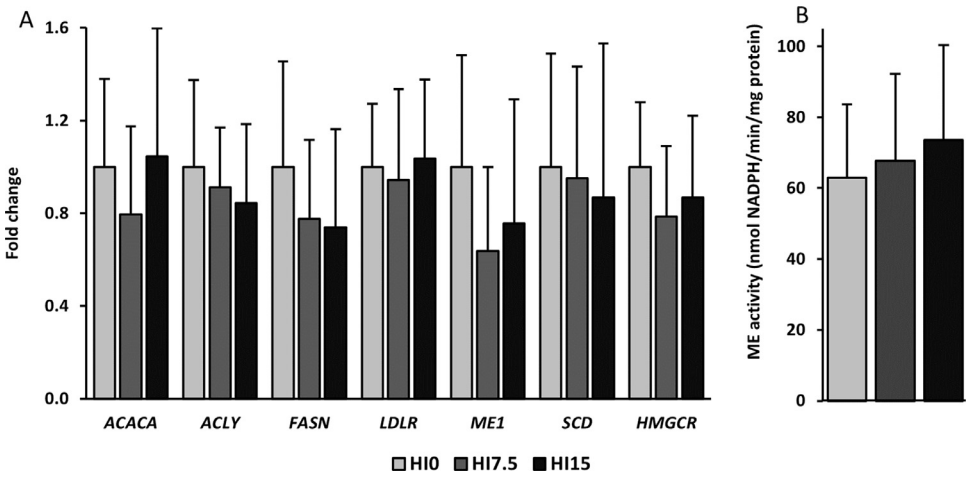


**Figure 4.** (A) Relative mRNA levels of lipogenic genes (*ACACA, ACLY, FASN, LDLR, ME1, SCD, HMGCR*) in the liver, and (B) ME activity in the cytosolic fraction of the liver of broilers fed diets with either 0% (HI0), 7.5% (HI7.5) or 15% (HI15) HI larvae meal for 35 d. Data are means ± SD for $n = 12$ broilers/group. Abbreviations: ACACA, acetyl-CoA carboxylase alpha; ACLY, ATP citrate lyase; FASN, fatty acid synthase; LDLR, low density lipoprotein receptor; ME1, malic enzyme 1; SCD, stearoyl-CoA desaturase; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase.

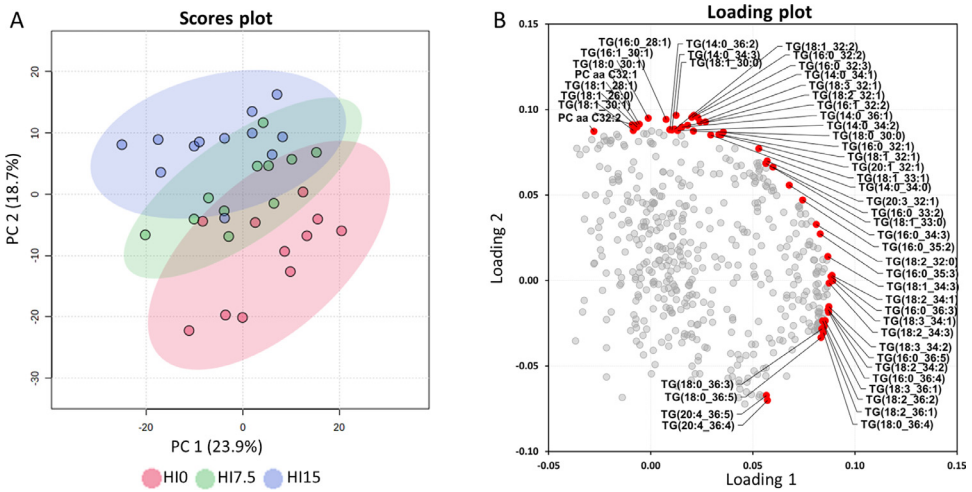


**Figure 5.** Principal component analysis of plasma metabolome. Scores plot with auto-scaling and log-transformation plotted with a 5% confidence interval (A) and the corresponding loading plot of plasma metabolome. Broilers were fed diets with either 0% (HI0), 7.5% (HI7.5), or 15% (HI15) HI larvae meal for 35 d. The principal components (PC) 1 and 2 explain together 42.6% of the variants in the data set. Parameters with the strongest effect are named. Data represent $n = 12$ broilers/group. Abbreviations: TG, triglycerides; PC aa, diacylphosphatidylcholine.

shows that the shift of the groups was mainly caused by certain TG species. The position of HI15 in the upper left corner of the scores plot was largely determined by TG species, such as TG(14:0_34:1), TG(16:0_32:2) and TG(16:1_30:1), with fatty acids C14:0 and C16:1, which are typical for HI, and TG species with monounsaturated fatty acids, such as TG(18:1_30:0) and TG (16:0_32:3). TG with polyunsaturated fatty acids, such as TG(20:4_36:4), TG(18:0_36:4, TG(18:0_36:5), TG (18:2_36:1), and TG(18:3_34:1), were responsible for the shift of group HI0 to the lower right corner of the scores plot.

## DISCUSSION

Despite the existence of a substantial number of studies investigating the effect of insect meal on the growth performance of broilers, knowledge about the metabolic effects of insect meal in broilers is still scarce. In order to close this gap of knowledge, the present study investigated the effect of partial replacement of soybean meal with HI larvae meal on the liver transcriptome, the plasma metabolome, and the cecal microbiota in broilers. To ensure that the changes observed were not caused by an unbalanced diet, the content of chitin-corrected CP, digestible amino acids, EE, and $AME_N$ were adjusted between the 3 diets within each phase.

Although the present study was not designed as a classical performance trial, the performance parameters were also recorded in the present study. An important finding of the present finding is that the breast muscle weight and the carcass yield were improved in broilers fed diets with 15% HI larvae meal. In the broilers fed the HI larvae meal at the lower inclusion level (7.5%), a numerical increase of their breast muscle weight and the carcass yield was found suggesting a positive impact of HI larvae meal on the performance of broilers even at a

relatively low dose. These findings are in line with several other studies showing an improvement of different performance parameters, like carcass yield and breast muscle weight, in broilers in response to replacement of different protein sources with HI larvae meal (Mohammed et al., 2017; Dabbou et al., 2018; Kareem et al., 2018; Attivi et al., 2020; Lalev et al., 2022). Some of the above studies also found an improved weight gain and feed conversion ratio, which was not the case in the present study. However, other studies observed either no effect (Onsongo et al., 2018; Elangovan et al., 2021) or even an impairment of different performance parameters following dietary replacement of conventional protein sources with HI larvae meal in broilers (Velten et al., 2018; Schiavone et al., 2019; Murawska et al., 2021), even at comparable replacement levels as in the present study. Although the reason for the different outcomes between the studies is unclear, it is a matter of fact that most of the abovementioned studies did not consider the chitin content of the HI larvae meal making it impossible to calculate diets with similar protein content at different inclusion levels of HI larvae meal. Despite the concentration of chitin-corrected CP was slightly higher in the 2 HI larvae meal-containing diets than in the HI0 diet, the calculated concentrations of digestible amino acids in the 3 diets were similar and sufficient to cover the requirement of broilers indicating that an increased supply of digestible amino acids is not responsible for the increased breast muscle weight of broilers of group HI15. Interestingly, a previous study in broilers demonstrated that feeding a diet rich in the medium-chain fatty acid (**MCFA**) lauric acid (C12:0) also caused an increase in the relative breast muscle weight indicating that MCFA promote breast muscle growth (Zeitz et al., 2015). In addition, an increased breast muscle weight was recently observed in broilers fed HI larvae fat containing nearly 60% of total fatty acids as lauric acid (Schäfer et al., 2023a). In the present study, the diets of groups HI7.5 and HI15 contained >4 and >9% of total fatty acids as lauric acid suggesting that the increased breast muscle weights of broilers in these two groups might be also caused by the intake of lauric acid. Regarding the underlying mechanism, it has been proposed that MCFA improve energy availability for tissue growth because TG-bound MCFA are absorbed more efficiently than TG-bound long-chain fatty acids due to an easier emulsification and less dependence on pancreatic lipase (Hanczakowska 2017), at least in piglets (Zentek et al. 2012).

Plasma metabolomics revealed changes in a large set of metabolites most of which were TG species and largely reflected the fatty acid profile of the diets, from which the diets of group HI15 had the highest concentrations of C12:0, C14:0, C16:0 and C16:1 whereas the diets of group HI0 had the highest concentrations of C18:1, C18:2 and C18:3. In line with this, the concentrations of TG species with ≤ 3 double bonds were highest in group HI15 followed by group HI7.5 and group HI0, and most of these TG species contained the HI larvae-specific fatty acids C12:0, C14:0 and C16:1. In contrast, the concentrations of highly unsaturated TG species with ≥ 5 double bonds were highest - with only few exceptions (e.g. TG(14:0_38:5) - in group HI0 followed by group HI7.5 and group HI15. Regarding TG species with 4 double bonds, the concentrations were highest in group HI0 and group HI15 depending on the fatty acid profile; that is, the concentrations of TG species with C14:0 and other HI larvae-typical fatty acids were highest in group HI15 and those with polyunsaturated fatty acids, like C20:4 and 18:3, were highest in group HI0. The total sum of TG and the sum of different TG subspecies (saturated, monounsaturated) in plasma did not differ among groups, which is in line with the result from plasma TG determination by the enzymatic reagent kit. Following TG, glycerophospholipids were the second most affected metabolite class identified by plasma metabolomics. Similar as for TG, the concentrations of glycerophospholipids with ≤ 3 double bonds were highest in group HI15 followed by group HI7.5 and group HI0. Changes in the plasma concentrations of other lipid classes, like cholesterol esters, fatty acids, sphingolipids, and ceramides, also reflected the fatty acid profile of the diets and followed the same pattern as described for TG and glycerophospholipids. Considering that carnitine supplementation was reported to improve breast muscle weight of broilers (Xu et al., 2003), elevated plasma concentrations of carnitine and acetylcarnitine in group HI15 compared to group HI0 could have contributed to the improved breast muscle weight in broilers fed the high dose of HI larvae meal. Future studies are warranted to address this issue.

Apart from lipids, plasma metabolomics revealed increased concentrations of several amino acids, such as alanine, serine, proline, and threonine, in groups HI15 and HI7.5 compared to group HI0, which may be the consequence of the above-described increased AID of amino acids. Increased plasma concentrations of certain amino acids, such as alanine, glutamate, proline, serine, tyrosine and valine, have been reported recently for pigs fed diets containing TM larvae meal (Meyer et al., 2020b). Besides amino acids, the plasma concentrations of several amino acid-related metabolites, like 1-methylhistidine, 3-methylhistidine, D-$\alpha$-aminobutyric acid, 3-aminobutanoic acid, betaine, proline betaine, sarcosine, $\beta$-alanine and methionine sulfoxide were found to be different between groups. Although the reasons for most of these changes are currently unknown, the increase of methionine sulfoxide in broilers fed the HI larvae meal has been also observed in other studies with rats (Gessner et al., 2019) and pigs (Meyer et al., 2020a) fed diets containing larvae meal from other insect species (TM). Regarding this and the fact that there were no indications of the induction of oxidative stress in broilers fed the HI larvae meal, it is likely that the increase of methionine sulfoxide in plasma is the result of ingestion of methionine sulfoxide from insect larvae meal. This assumption is supported by an earlier study reporting the detection of methionine sulfoxide in *Sitophilus oryzae* larvae (Gasnier-Fauchet and Nardon, 1987).

A further finding of the present study is that the impact of soybean meal replacement by HI larvae meal in the diets on the liver transcriptome of broilers is very weak. This was evident from the low number of differentially regulated genes (70 for the comparison HI15 vs. HI0 and 61 for the comparison HI7.5 vs. HI0) and the generally weak regulation of genes; only 2 genes were found to be regulated ≥ 2-fold (*DDX60, ETNPPL*) at the higher dose of HI larvae meal and none at the lower dose of HI larvae meal. One of these, *DDX60*, belongs to the family of DEAD/H-box RNA helicases which participate in anti-viral innate immunity, either by acting as viral nucleic acid sensors or by regulating downstream signaling events (Fullam and Schröder, 2013). In line with a role in innate immune activation, *DDX60* was found to be up-regulated in tonsils and intestine of chickens following infection with avian influenza virus H9N2 (Alqazlan et al., 2021), and in the spleen of chickens infected with the commonly observed chicken astrovirus CAstV (Sajewicz-Krukowska et al., 2021). The other gene, *ETNPPL*, being highly expressed in liver and brain encodes the enzyme ethanolamine phosphate phospholyase, which specifically degrades phosphoethanolamine, which itself is necessary to generate phosphatidylethanolamine *via* the so-called Kennedy pathway (Pavlovic and Bakovic, 2013). According to recent research, *ETNPPL* is involved in palmitic acid-induced hepatic insulin resistance (Wang et al., 2023). However, the biological relevance of the moderate up-regulation of both, *DDX6* and *ETNPPL*, in the liver of broilers fed HI larvae meal is unclear and needs to be elucidated in future studies. The most strongly, even though weakly down-regulated gene was *STARD4* (FC -1.67), which encodes a protein mediating the delivery of cholesterol into the mitochondrial membrane. Recently, the sterol regulatory element-binding protein 2 (SREBP2) − the master regulator of cholesterol homeostasis in animals − was described to be a direct transcriptional regulator of *STARD4* (Yue et al., 2023). This finding might suggest that HI larvae meal affects cholesterol homeostasis in the liver of broilers. In line with this, *HMGCR* − a further well-known target gene of SREBP2 encoding the rate-limiting enzyme of cholesterol synthesis - and *INSIG1*, which plays a role in the proteolytic generation of the transcriptionally active nuclear forms of SREBF, were also identified as genes being weakly down-regulated (−1.44 and −1.35, respectively) in the HI15 group compared to the HI0 group. However, measurement of cholesterol concentrations in liver and plasma of the broilers revealed no difference between groups indicating that the weak effects of dietary HI larvae meal inclusion on the hepatic transcriptome were not associated with phenotypic alterations. In line with the overall weak regulation of the hepatic transcriptome by dietary HI larvae meal, bioinformatic analysis of microarray data revealed only very few enriched GO biological process terms within the genes differentially regulated between groups fed HI larvae meal and group HI0. Given this, the biological processes identified to be affected in broilers fed HI larvae meal should not be overinterpreted.

One effect observed in the present study that might receive some attention is the increase of hepatic TG concentration in broilers of group HI15, because hepatic lipid accumulation is known to be associated with an impaired liver function (Hu et al., 2023). This result was partially confirmed by fatty acid analysis of hepatic total lipids in that the sum of total fatty acids tended to be higher in group HI15 than in group HI0. However, apart from the fact that fatty acid analysis of hepatic total lipids revealed higher proportions of HI larvae-typical fatty acids, like C12:0 and C14:0, and lower proportions of C18:0, C18:3 n-6, C20:4 n-6 and C24:1 n-6 in the HI15 group compared to the HI0 group, there were no alterations in the proportions of fatty acids originating mainly from de novo-fatty synthesis, like C16:0 and C16:1. Together with the largely unaltered hepatic expression of hepatic lipogenic genes, which was evident from both microarray analysis and qPCR, suggests that HI larvae meal does not stimulate hepatic lipid synthesis.

Analysis of the cecal microbiota revealed an increase of several indicators of α-diversity (Chao1, Observed, Shannon) in the cecum of broilers of group HI15 compared to group HI0 suggesting that both, species richness and diversity were increased by dietary HI larvae meal inclusion. An increase of gut microbial diversity, which has been also observed in broilers fed diets with TM larvae meal (Biasato et al., 2018b; Benzertiha et al., 2019; Józefiak et al., 2020), is generally considered beneficial, because a more diverse microbiota is known to be more resilient to exogenous stimuli, such as pathogens. Changes in the taxonomic composition of the cecal microbial community were mainly observed at the family (Paenibacillaceae, Ruminococcaceae, Clostridia vadinBB60 group, uncultured Oscillospirales, Butyricicoccaceae), and the genus level and occurred exclusively within the Firmicutes, which was the by far most dominant phylum in the cecum of all groups. The abundance of most of the differential bacterial families including the Ruminococcaceae were increased in the HI15 and HI7.5 groups compared to the HI0 group. This effect of dietary inclusion of HI larvae meal is possibly beneficial considering that a decreased abundance of Ruminococcaceae has been implicated in inflammatory bowel diseases in humans (Joossens et al., 2011). In contrast, the reduction of the abundance of Butyricicoccaceae - a family comprising butyrate-producing taxa - in the HI15 group compared to the HI0 group could be interpreted as less favorable, because butyrate is known to strengthen the gut barrier. At the genus level, several beneficial effects were seen, such as an increased abundance of *Lachnoclostridium* in the cecum of broilers of the HI15 group. *Lachnoclostridium* is known to inhibit the proliferation of pathogens and alleviates intestinal inflammation, and its abundance is associated with anti-inflammatory effects in the intestine of broilers (Shang et al., 2020). In contrast, the abundance of *Shuttleworthia*, a potential pathogen, whose abundance was reported to be increased during intestinal infections in broilers (Chen et al., 2020; Khan and Chousalkar, 2020), was decreased in the cecum of broilers of the HI15 group. The

decreased abundance of *Oscillibacter* in the cecum of group HI15 is also a beneficial effect considering that this genus was found to be related to gut permeability and intestinal inflammation (Li et al., 2021). Considering that some of the effects on the cecum microbiota discussed indicate a positive effect of dietary HI larvae meal inclusion on the gut microbiota diversity and composition of the broilers, it is possible that these effects have contributed to the improved breast muscle weights. This assumption is based on the knowledge that gut integrity is markedly influenced by the gut microbial community and gut integrity is a key prerequisite for animal health and performance (Ringseis and Eder, 2022). Despite the reason for the modulatory effect of dietary HI larvae meal inclusion on the cecal microbiota of the broilers is unknown, it is possible that different compounds which are contained in HI larvae meal, such as chitin, lauric acid and antimicrobial peptides, and have been reported to alter the gut microbiota structure of broilers (Zeitz et al., 2015; Zhang et al., 2020; Wang et al., 2023), are causative.

A change of the gut microbiota structure is commonly associated with altered concentrations of SCFA in the gut due to differences in the fermentation pathways utilized by different bacterial taxa. In the present study, the cecal concentration of butyric acid differed between the HI15 group and the HI group, whereas cecal concentrations of total SCFA and all other individual SCFA were not different between the HI15 group and the HI0 group. The reduction in the concentration of butyric acid in the HI15 group compared to the HI0 group is likely reflective of the above-described changes of the cecum microbiota structure, because the bacterial taxa Butyricicoccaceae, *Shuttleworthia, Eubacterium halli group* and *Butyricicoccus*, whose abundance in the cecum was reduced in the HI15 group compared to the HI0 group, are well-known to produce butyrate as the major fermentation product (Duncan et al., 2004; Louis and Flint, 2009; Geirnaert et al., 2014).

## CONCLUSION

Comprehensive analysis of the metabolic effects of replacement of soybean meal with HI larvae meal in the diets of broilers using liver transcriptomics, plasma metabolomics, and cecal microbiota analysis shows no negative impact of dietary HI larvae meal but beneficial increases of breast muscle weight and carcass weight and an improvement of the AID of several amino acids. Based on these findings HI larvae meal can be recommended as a sustainable alternative protein source for broilers.

## ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (DFG; Grants RI 1537/9-1 and ED 70/15-1).

## DISCLOSURES

The authors declare that they have no competing interests.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.103635.

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