Full-fat insect meal in pelleted diets for weaned piglets: effects on growth performance, nutrient digestibility, gastrointestinal function, and microbiota

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Abstract

Insects, such as the black soldier fly larvae (BSFL), are suggested as a sustainable novel protein source for pigs. The BSFL contains chitin, medium chain fatty acids, and anti-microbial peptides, which could improve the gastrointestinal function and health of the post-weaning pig. The objective of this study was to investigate the effect of increased inclusion of full-fat BSFL in diets for post-weaning pigs on growth performance parameters, digestibility of nutrients, gut morphology, and the microbial community in the colon. Eighty crossbred pigs were weaned at approximately 32 days of age, with an average weaning weight of 10.6 ± 0.8 kg. For four weeks, pigs were fed: a control diet or one of three diets containing increasing amount of full-fat BSFL meal at 4.76%, 9.52%, and 19.06%. The ADG for the overall experimental period showed a negative cubic effect of dietary treatment, where the ADG was highest for pigs fed the control diet and lowest for pigs fed the BSFL5. Increased level of full-fat BSFL in the diet did not affect feed efficiency or fecal consistency. A linear reduction in the coefficient of total tract apparent digestibility (CTTAD) of crude protein ($P = 0.011$) was found for increasing inclusion of BSFL, whereas for crude fat both the coefficient of ileal apparent digestibility ($P = 0.043$) and the CTTAD ($P<0.001$) increased linearly. Jejunal, ileal, or colonic morphometry was not affected by the BSFL inclusion. No differences in the short chain fatty acid concentrations were detected among the dietary treatments, but a few minor changes in the colon microbiota were observed. At the phylum level, the colon microbiota was dominated by Bacteroidota and Firmicutes, but there was no clear pattern relationship with the BSFL inclusion level. At the genus level, the inclusion of BSFL in the diet reduced the relative abundance of *Lactobacillus* ($P = 0.015$) compared to the control. Collectively, the results indicate that up to 19.06% of full-fat BSFL meal could be included in a

balanced diet for PW pigs with only minor effects on growth performance, general gut function, and gut health.

Keywords: Pig, Weaning, Insect meal, Black soldier fly larvae, Gut function, Microbiota

Abbreviations

ADF, acid detergent fiber; ADFI, average daily feed intake; ADG, average daily gain; aNDF, amylase-treated neutral detergent fiber; ASV, amplicon sequence variant; BSFL, black soldier fly larvae; CD, crypt depth; CF, crude fat; CIAD, coefficient of ileal apparent digestibility ; CISD, coefficient of ileal standardized digestibility; CP, crude protein; CTTAD, coefficient of total tract apparent digestibility; DM, dry matter; FTIR, Fourier Transform Infrared Imaging; G:F, gain:feed ratio; GIT, gastrointestinal tract; MCFA, medium chain fatty acid; PCoA, principal coordinate analyses; PW, post-weaning; SCFA, short chain fatty acid; SEM, standard error of the mean; VH, villus height.

1. Introduction

The world is facing a growing food demand by an ever-increasing number of people. To meet this challenge, new resources must be considered by using innovative solutions. Insects have been proposed as a high quality, efficient, and sustainable alternative protein source (Veldkamp et al., 2012). Black soldier fly larvae (BSFL), *Hermetia illucens*, is an easily reared species, capable of efficient conversion of a wide range of organic materials (Wang and Shelomi, 2017), and do not accumulate pesticides or mycotoxins (Purschke et al., 2017; Leni et al., 2019). The BSFL has an

overall favorable amino acid profile but is limiting in sulfur-containing amino acids and contains a high amount of minerals, especially calcium (Finke, 2013).

A well-functioning gastrointestinal tract (GIT) is of importance to the overall growth performance and health of pigs in all stages, especially for the newly weaned pigs. If not defatted, BSFL is high in fat, especially in lauric acid (12:0; Finke, 2013) which is categorized as a medium chain fatty acid (MCFA) with anti-microbial effects, especially against gram-positive bacteria (Zentek et al., 2011). Insects and insect larvae also contain chitin, a dietary polysaccharide that can function as a prebiotic and an immunostimulant (Song et al., 2014). Finally, anti-microbial peptides, which are part of the insect immune system, are effective anti-microbial agents with low risk of development of bacteria resistance (Lewies et al., 2019). The anti-microbial peptides have good potential as health promoters in livestock, even though there is limited information about the *in vivo* effects (Wang et al., 2016).

Insects are therefore interesting to investigate both as a feed ingredient and as a functional ingredient with potential health beneficial effects. Recently there has been a high focus devoted to the potential of insect meals in diets for monogastric farm animals (Józefiak et al., 2018; Spranghers et al., 2018; Biasato et al., 2019; Nogales-Mérida et al., 2019), however, there is a scarcity of literature on the use of full-fat BSFL in diets for pigs. The objective of this study was to investigate the effect of increasing inclusion of full-fat BSFL in diets for post-weaning (PW) pigs, focusing on growth performance parameters, nutrient digestibility, gut morphology, and microbial community in the colon.

2. Materials and Methods

A 27-day experiment was performed in February 2019 at the Center for Livestock Production (SHF, Norwegian University of Life Sciences, Ås, Norway), which is an animal experimental unit approved by the National Animal Research Authority (permit no. 174). All pigs were handled under the applicable laws and regulations controlling experiments with live animals in Norway regulated by the "Animal Welfare Act" and "The Norwegian Regulation on Animal Experimentation" derived from the "Directive 2010/63/EU on the protection of animals used for scientific purposes".

2.1 Animals and Housing

Eighty crossbred [Norwegian Landrace x Yorkshire z-line) x Duroc)] weaned pigs were included in the experiment. All piglets had access to the sows feed during the nursing period. The experiment was conducted as a randomized complete block design. Piglets were selected from eleven litters (four or eight pigs from each litter depending on the litter size), based on their weaning weight to create a uniform group, and then equally distributed to the four dietary treatments based on litter, sex and weight. The average weaning age was 32.8 ± 1.6 days and the average weaning weight was 10.6 ± 0.8 kg. There were five pens per treatment with four pigs per pen, containing two gilts and two barrows. No pigs in the same pen were siblings. Three of the pens per treatment were installed with rubber mats. The remaining pens had wood shavings as bedding material. The pen size was 1.6 m^2. The room temperature was logged every morning, and the average temperature for the experimental period was 21.6 ± 1.4 °C.

2.2 Dietary treatments

BSFL meal was produced at HiProMine S.A., Poznan, Poland. The BSFL feed had a DM level of 22% and consisted of 17% wheat middlings and 83% fresh vegetable mix, consisting of apples (15%) carrots (50%) potatoes (15%) and cabbage (20%). Fresh vegetable pre-consumer waste was ground (2000 rpm/1 min, (HPM milling system, 55 kW, Poland) to pass a 2 mm screen and offered *ad libitum* to the BSFL. Substrates were not contaminated by any animal products in accordance with EC regulation (no 1069/09). At the prepupal stage (10th day of rearing), larvae were harvested, sieved through a 3 mm screen, and washed with water on drum separator at 90 ºC for 10 minutes (HPM cleaning system, Poland).

The dietary treatments included a control diet and three experimental diets with increasing inclusion of BSFL; 4.76% (BSFL5), 9.52% (BSFL10), and 19.06% (BSFL20; Table 1). Diets were formulated in collaboration with Felleskjøpet Fôrutvikling AS (Trondheim, Norway) using their optimization least-cost program based on the Dutch energy evaluation system (Blok et al., 2015). Diets were formulated based on net energy and coefficients of ileal standardized digestibility (CISD) to be isoenergetic, balanced for digestible amino acids, and to meet or exceed the nutrient requirements of pigs (NRC, 2012). Literature values from Finke (2013) for the amino acid content in the BSFL meal were used in the diet formulation. CISD for the amino acids in the BSFL meal were set to 0.83. Yttrium oxide (Y_2O_3) was included as an inert marker in the diets (0.01 %) for digestibility calculations. Pelleted diets were produced by the Center for Feed Technology (FôrTek, Norwegian University of Life Sciences, Ås, Norway). The feed mash was ground in a Münch hammer mill (HM 21.115, Wuppertal, Germany) fitted with a 3 mm screen before pelleting. The mash was steam conditioned at 82 °C in a double-pass pellet-press conditioner

(Münch-Edelstahl, Germany) before pelleting (Münch-Edelstahl, Germany, 2 × 17 kW) through a 3.5 mm die with a production rate of 700 kg/h. Pigs had *ad-libitum* access to the experimental diets immediately after weaning through automatic feeders (FRH-2 Domino A/S, Tørring, Denmark) with 43 cm feeding space. The automatic feeders were checked daily and refilled when needed. Feed residues were registered weekly and average daily feed intake (ADFI) per pen was calculated. Clean drinking water was always available from a drinking nipple next to the feeder.

The farm had an ongoing issue with edema disease and symptoms resulted in antibiotic treatment of five pigs on days 8-10 PW. After one pig died without any registered symptoms on day 11 PW, all pigs were treated with intramuscular antibiotic injections (Borgal vet., Ceva Santé Animale, Libourne, France) for three consecutive days (11-13 PW). After treatment, all pigs appeared healthy throughout the experiment.

2.3 Sample collection

Fecal consistency was assessed daily and registered for all pens based on the four category scale developed by Pedersen and Toft (2011). A higher score indicated more watery feces. Scores one and two were considered normal while scoring three and four were considered as diarrhea. The daily fecal score was registered as a pen average with 0.25 intervals on the scale. All pigs were weighed weekly and average daily gain (ADG) and gain:feed ratio (G:F) was calculated per pen. Fecal samples were also collected every week for the determination of the fecal DM. An approximately equal amount of feces from each pig was pooled for the pen before oven drying at 103 °C for 24h. Fresh individual fecal samples were collected on days 21, 22, 25, 26, and 27 for determination of the coefficient of total tract apparent digestibility (CTTAD) of nutrients.

Individual samples from all days were pooled, freeze-dried, and ground using a Retsch ZM 100 centrifugal mill (Retsch, Haan, Germany) fitted with either a 0.5mm or a 1mm screen before chemical analysis. Apparent digestibility of nutrients was calculated as described by Maynard and Loosli (1969).

2.4 Terminal sample collection

Only pigs from the pens with rubber mats (n = 3 pens per treatment, a total of 47 pigs) were included in the terminal sampling at day 28 and 29 PW. Pigs were fasted from the evening before but had access to feed three hours before euthanasia. Euthanasia was done using a captive bolt pistol followed by exsanguination. Immediately after exsanguination, the abdominal cavity was opened, and the GIT was removed. pH was measured in stomach and jejunal content. Intestinal content was collected from jejunum (first 30 cm post the hepatopancreatic duct) and from the spiral colon. Tissue from oral jejunum (15 cm post the hepatopancreatic duct), aboral ileum (15 cm from the ileocecal valve), and the spiral colon were collected for histological assessment. Intestinal content from the last two meters of the small intestine was collected and stored at -20°C for determination of the coefficient of ileal apparent digestibility (CIAD) of nutrients. For seven of the pigs (three control pigs, two pigs fed BSFL10, and two pigs fed BSFL20), samples were incorrectly collected from jejunum instead of ileum, and therefore excluded from analyzes. The ileal contents were freeze-dried and homogenized using a batch mill (A11 basic Analytical mill, IKA, England). Liver weight was recorded to calculate liver index: [liver weight (kg) / live body weight (kg) * 100].

2.5 Chemical analyses

Pooled feed samples for each diet, collected from the feed storage during the experiment when preparing feeding, were ground using a Fritsch Pulverisette 19 cutting mill (Fritsch GmbH, Idar-Oberstein, Germany) fitted with either a 0.5mm screen or a 1.0mm screen before the chemical composition of nutrients were analyzed in triplicates (Table 2, 3, and 4). The chemical analyses were performed by the LabTek group at the Department of Animal and Aquacultural Science, Norwegian University of Life Sciences, Ås, Norway. The DM content was determined by drying to constant weight at 103 °C ± 2 °C (ISO 6496, 2001), and ash was determined by complete combustion at 550 °C for at least 4h (ISO 5984, 2002). Gross energy (GE) content was determined using a PARR 6400 Automatic Isoperibol Calorimeter (Parr Instruments, Moline, IL, USA) according to ISO 9831 (1998). Crude protein (CP) was analyzed with the Kjeldahl method according to Commission Regulation (EC) No 152/200, using a Digestor 2520 (FOSS Analytical, Hillerød, Denmark) and the Kjeltec 8400 analyzer (FOSS Analytical, Hillerød, Denmark). Crude fat (CF) was analyzed using Accelerated Solvent Extraction (ASE 350, Thermo Fisher Scientific, Waltham, MA, USA). Extraction was conducted with 80% petroleum ether and 20% acetone at 125 °C. Starch was determined using an enzymatic-colorimetric method according to McCleary et al. (1994), with some modifications. In brief, starch was degraded with heat-stable α-amylase and amyloglucosidase-enzymes to glucose. Glucose concentration was then determined using a spectrophotometer (RX Daytona +, Randox Laboratories Ltd., Crumlin, UK). Acid detergent fiber (ADF) and amylase-treated neutral detergent fiber (aNDF) were analyzed using the Ankom200 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA). For aNDF analysis, the sample was treated with α-amylase and analyzed according to Mertens (2002). To determine ADF content, samples was digested with 1.00 N sulfuric acid containing 20 g cetyl trimethylammonium bromide

according to the manufacture's instruction. Amino acids in diets and ileal samples were analyzed according to Commission Regulation (EC) No 152/2009. Amino acids were analyzed on a Biochrom 30+ Amino Acid Analyzer with an autosampler (Biochrom Ltd., Cambridge, UK). Tryptophan was analyzed in diets on a Dionex Ultimate 3000 HPLC system (Dionex Softron GmbH, Germering, Germany) with a Shimadzu RF-535 fluorescence detector (Shimadzu Corporation, Kyoto, Japan). The fatty acid composition of the diets was analyzed according to O'fallon et al. (2007) by synthesizing the fatty acids to fatty acid methyl esters (FAME), in which concentrations were determined using a Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA). Total phosphorus was analyzed after combustion and acid digestion (Commission Regulation (EC) No 152/2009) using a commercial spectrophotometric kit (PH8328, Randox laboratories, County Antrim, UK). Yttrium concentration was determined after acid decomposition in a microwave digestion system (Start D, Milestone S.r.l., Sorisole, Italy), using a microwave plasma atomic emission spectrometer (MP-AES 4200, Agilent Technologies, Santa Clara, CA, USA). The chitin content of the BSFL meal was determined according to Finke (2007).

Trypsin and lipase activities were analyzed in jejunal content. Ice-cold Milli-Q water (1.5 ml; 600 µl for lipase analysis) was added to approximately 100 mg of the jejunal content, homogenized using a bead mill (TissueLyser, Qiagen Retsch, Haan, Germany) and sonicated in an ice-cold bath for three minutes (T 460/H, Elma Schmidbauer GmbH, Ransbach-Baumbach, Germany). After centrifugation at 21,100 ×*g* for 10 min at 4 °C, the supernatant was collected, aliquoted, stored at -80 °C, and used for the analysis of lipase, trypsin, and total protein. Total protein concentration was determined in microtiter assay according to the Quick Start Bradford Protein Assay protocol

(Bio-Rad Laboratories, Oslo, Norway). Absorbance was measured using a SpectraMax M2e Microplate Reader (Molecular Devices, LLC., San Jose, CA, USA). Lipase and trypsin activity were analyzed using commercial kits (Lipase Activity Assay Kit III, MAK048-1KT, fluorometric, Sigma-Aldrich, Merck KGaA; Trypsin Activity Assay Kit, ab102531, colorimetric, Abcam), according to the manufacturer's protocols.

Short chain fatty acids (SCFA) were analyzed in colon content. Samples were thawed on ice and 500 mg of the colon content were mixed with 500 µl ice-cold internal standard solution (2-methyl valeric acid in 5% formic acid), before sonication for 5 min in cold water. After centrifugation for 15 min at 4 °C with 15000 ×*g*, the supernatant was transferred to a spin column (45 kDa; VWR International, Radnor, PA, USA) and centrifuged again with the same parameters. SCFA concentration was determined by capillary gas chromatography on a stabilwax-DA, 30 m × 0.25 mm × 0.25 µm capillary column (Restek Corporation, Bellefonte, PA, USA) installed on a Trace 1300 gas chromatograph equipped with an AS 1310 autosampler, split injection, a flame ionization detector and Chromeleon software (Thermo Fisher Scientific, Waltham MA, USA). The initial oven temperature was 90 °C, held for 2 min, followed by a temperature increase of 10 °C/min to 150 °C and 50 °C/min to 250 °C and then held for 1 min. Helium was used as the carrier gas at a flow rate of 3 mL/min. The injector temperature was set at 260 °C and the detector temperature was set at 275 °C.

2.6 Morphology and morphometry

Jejunal, ileal, and colon morphology were blindly assessed and villus heights (VH) and crypt depths (CD) were measured by Aquamedic AS, Oslo, Norway. Gross pathological observations

were recorded before tissue sampling. Samples were fixated in 10% formalin up to 48 h before processing following standard histological methods for gut tissue. Sections were stained with hematoxylin and eosin and evaluated by light microscopy, where the evaluation was done on morphological characteristics such as epithelial cell and barrier morphology and integrity, crypt changes such as hyperplasia, dilation or abscessation, degenerative and inflammatory mucosal changes including increased numbers of intraepithelial lymphocytes and infiltration by leucocytes. Methodologies of the evaluation protocol are described by Day et al. (2008) and Pérez de Nanclares et al. (2017). The morphological characteristics evaluated were graded using a semi-quantitative scoring system where score 0 is normal, score 1 represent mild changes, and score 2, 3, and 4 represent moderate, marked, and severe changes, respectively. VH and CD measurements were made on scanned whole-section images of the respective tissues captured using the PreciPoint M8 Microscope and Scanner (PreciPoint, Freising, Germany), and obtained using the ViewPoint software (PreciPoint, Freising, Germany). A minimum of three well-oriented crypts and villi were measured from each of the sections.

2.7 Extraction of DNA and 16S rRNA sequencing

Total DNA was extracted from bacteria in approximately 190 mg of colon content using QIAamp Fast DNA Stolen Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. The DNA concentration was determined using NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All samples were stored at -20 ºC until further analysis. Before preparing for 16S rRNA sequencing, samples were normalized to 10 ng/µL. The V3-V4 regions of the bacterial 16S rRNA gene were amplified using the primers Pro341f (5'-CCTACGGGNBGCASCAG-3') and Pro805r (5'-GACTACNVGGGTATCTAATCC-3'). The

library preparation was conducted using the Miseq Reagent Kit V3 (Illumina, San Diego, CA, USA) according to the Illumina 16 S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA). For indexing, Nextera XT index kit V2 was used (Illumina, San Diego, CA, USA). After the indexing reaction, all samples were measured by Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) using Qubit 1X dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA). An equal amount of each sample was pooled together, and spiked with 5% PhiX Control (Illumina, San Diego, Waltham, MA, USA). For sequencing, 10 pm of the pooled sample was loaded to a flow cell. The sequencing analysis was performed on the Miseq System (Illumina, San Diego, CA, USA). The clustering density was 1256 k/mm2 and 88.3% of clusters were passing filter.

Raw sequence data were analyzed using DADA2 v. 1.12.1 (Callahan et al., 2016) in R v. 3.5.0 (R Core Team, 2019). Default parameters were used if other is not specified. In brief, primers were removed, and the forward reads were truncated at 280 bp and the reverse reads at 250 bp. Max expected errors were set to 7. This allowed 73% of the reads to pass the quality control. Error rates were estimated, and the core sample inference algorithm applied. The denoised read pairs were merged, and chimeras removed. The Silva v. 138 database (Quast et al., 2013; Yilmaz et al., 2014) was used as a reference database for the taxonomy assignment. A phyloseq object was built with the phyloseq v.1.26.1 for further analyses (McMurdie and Holmes, 2013). Figures were made with ggplot2 v.3.2.1 (Wickham, 2016).

2.8 Statistical analysis

Outliers in the data were identified using the interquartile range (IQR) method. Values outside the range of three times the IQR outside the lower and higher quartile were defined as outliers. Results are presented as mean values and standard error of the mean (SEM). Statistical analysis was performed using R v.4.0.3 (R Core Team, 2019) in Rstudio v.1.3.1093 (Rstudio, Boston, MA, USA). A linear mixed-effects procedure was run, using the lme4 1.1-23 (Bates et al., 2015) and lmerTest 3.1-2 (Kuznetsova et al., 2017) packages. Initially, the following model was used for variables from individual samples (pH, digestibility, enzyme activity, intestinal morphology and morphometry, liver index and SCFA):

$Y_{ijklm} = \mu + diet_i + sex_j + bedding_k + litter_l + \varepsilon_{ijklm}$ where Y is one observation on pig n; μ is the intercept; $diet_i$ is the fixed dietary treatment effect (i = 1:4); sex_j is the fixed effect of the sex of the pig (j = 1,2); $bedding_k$ is the fixed effect of bedding material, rubber mat or wood shavings (k = 0,1); $litter_l$ is the random effect of the lth litter (l = 1:11) $\sim N(0, \sigma^2_{litter})$ and ε_{ijklm} is a random residual $\sim N(0, \sigma^2_{\varepsilon})$. Pen (1:20) $\sim N(0, \sigma^2_{pen})$ was considered as a random effect in the model, but the effect of pen was tested with a likelihood-ratio test and found insignificant ($P > 0.05$) for all the variables. Hence, the pen was removed from the final model. An orthogonal polynomial contrast matrix, adjusted for BSFL inclusion levels, was specified using the contr.poly function in the stats base package (R Core Team, 2019). The bedding was not included in the model when analyzing the terminal sampling parameter, as it was constant. Effects are considered statistically significant when $P < 0.05$ and tendencies are defined as P-values between 0.05 and 0.10.

For the effects of sex and bedding material, only significant results are presented.

For all pen-level parameters (growth performance, fecal DM and fecal score), the following model was used with the lm function in the stats base package: $Y_{ikn} = \mu + diet_i + bedding_k + \varepsilon_n$ where Y now is one observation on pen n.

The Fisher's exact test was used to analyze data from the macroscopic evaluation of the intestinal segments. Histopathological findings was analyzed using the Kruskal-Wallis Rank Sum Test in the stats package (R Core Team, 2019).

To assess diversity in the microbial communities within pigs, Shannon alpha-diversity indices were calculated at the amplicon sequence variant (ASV) level. A comparison of the Shannon indices among dietary treatments was done using the Kruskal-Wallis Rank Sum Test. The beta-diversity (among pig variation in microbial communities) was assessed by principal coordinate analyses (PCoA) with the Bray-Curtis, unweighted, and weighted UniFrac distance matrices. Beta dispersion (variances) was calculated within each dietary group and a permutation-based test of multivariate homogeneity applied (vegan package v.2.6-6; Oksanen et al., 2019). Fulfilling the assumption of homogeneity in group variance, a PERMANOVA test was performed on the distance matrices with the dietary group as the independent variable. Pairwise PERMANOVA tests were performed to compare beta diversity among dietary treatments. P-values were adjusted for multiple testing with the method by Benjamini and Hochberg (1995).

The relative abundance was calculated, and the statistical difference in relative abundance among dietary treatments was analyzed with the Kruskal-Wallis test with dietary treatment as the explanatory variable.

To test for a statistical difference in relative abundance among dietary treatments, the Kruskal-Wallis test with dietary treatment as the explanatory variable was applied. Two-sample Wilcoxon

tests, also known as the Mann-Whitney test, was used for pairwise comparison between the diets if the Kruskal Wallis test gave a significant effect of dietary treatment. P-values were corrected for multiple testing with the Benjamini and Hochberg (1995) method.

To test for covariation between the microbiota and SCFA profile, dissimilarity indices with the Bray-Curtis indices were calculated separately for the microbiota and SCFA data with the vegdist function, and the covariation tested with the mantel function in the vegan package (Oksanen et al., 2019).

3. Results

3.1 Growth performance

The ADG for the overall experimental period showed a negative cubic effect of dietary treatment, where the ADG was highest for pigs fed the control diet and lowest for pigs fed the BSFL5 (Table 5). The same tendencies were found for the ADG 0-14 days and 14-27 days, and for the final BW. There was also a tendency for increased G:F ratio for day 0-14 PW with increasing inclusion of BSFL. The ADFI was not affected by dietary inclusion of BSFL. Pigs in pens with wood shavings had higher ADG ($P = 0.040$) for day 14-27 PW.

3.2 General intestinal function

There were no differences in pH in stomach or pH in among the dietary treatments (Supplementary Table 2). There were no differences in fecal DM or fecal score (Table 6) among the dietary treatments. The graph of the overall development in the fecal score during the experimental period (Supplementary Figure 1) shows an increased score (>2) in the first week PW, which seems to

stabilize below score two after day 12, but again increasing to score >2 on day 23-25. Pens with wood shavings had lower fecal scores in week 3 (P = 0.049), and numerical lower average fecal score all experimental weeks.

3.3 Digestibility and enzymatic activity

There were no differences in jejunal trypsin or lipase activity among the dietary treatments (Supplementary Table 2). The CIAD of crude fat increased with increasing inclusion of BSFL in the diet, whereas the BSFL inclusion level had a positive quadratic effect on the CIAD of ash. No differences among dietary treatments were found for the CIAD of the other main nutrients (Table 7). Gilts had higher CIAD of CP (P = 0.030), CF (P = 0.025) and ash (P = 0.028), compared to barrows.

The CIAD of lysine was linearly reduced with increased inclusion of BSFL. A positive quadratic relationship was found between the BSFL inclusion level and CIAD of histidine, isoleucine, leucine, and phenylalanine. For the CIAD of tyrosine, there was both a linear decrease and a positive quadratic effect of the BSFL inclusion level. For the CIAD of the other amino acids and total amino acids, no differences were found among the dietary treatments (Table 8). An effect of sex was found for alanine (P = 0.013), arginine (P = 0.004), aspartic acid (P = 0.007), histidine (P = 0.008), isoleucine (P = 0.007), leucine (P = 0.009), lysine (P <0.001), methionine (P = 0.009), phenylalanine (P = 0.012), serine (P = 0.004), threonine (P = 0.007), valine (P = 0.013), and total amino acids (P = 0.022). All higher for gilts compared to barrows.

The CTTAD of CP was linearly decreased with increasing inclusion of BSFL, whereas by contrast, the CTTAD of CF and phosphorus increased (Table 9). The CTTAD of starch also tended to

increase with higher BSFL inclusion level. A linear and positive quadratic relationship was found between BSFL inclusion level and CTTAD of ash, where the CTTAD of ash was reduced compared to control, when adding 5% BSFL, but increased with BSFL inclusion level. Gilts had higher CTTAD of CP ($P = 0.035$), starch ($P = 0.015$) and phosphorus ($P = 0.005$). Using wood shavings as bedding material in the pens gave higher CTTAD of CP ($P = 0.035$), CF ($P = 0.001$), ADF ($P = 0.002$) and ash ($P = 0.010$).

3.4 Intestinal morphology, morphometry, and liver index

Macroscopic evaluation of the intestinal tissue during sampling revealed some changes, mostly in jejunum and colon, with reddening of the mucosa, and low muscle tone and no mucosal folding in the jejunum (Table 10, Figure 1). The macroscopic appearance was distributed equally with no clear differences among pigs fed the different dietary treatments.

Several pigs had morphological changes in jejunum. Occurrence of enterocyte vacuolization in jejunum differed among dietary treatments ($P < 0.001$), where all control pigs scored “normal”, whereas pigs fed the highest inclusion level all scored “mild”. Six pigs fed the BSFL5 and five pigs fed BSFL10 scored “mild”. In addition, three pigs fed the BSFL10 scored “moderate”. The remaining pigs scored normal. For the number of intraepithelial lymphocytes, all pigs scored normal except two pigs in the control group ($P = 0.082$). Dietary treatment did not affect lamina propria lymphocyte infiltration ($P = 0.899$), lamina propria edema ($P = 0.311$), or submucosal edema ($P = 0.581$) in jejunum. In the ileum, all tissue sections were observed to have a normal and healthy mucosal appearance.

406 In the colon, mild to moderate inflammatory morphological changes were observed. The
407 inflammation was largely characterized by an increased content of lymphocytes and plasma cells
408 in the inter-crypt compartment (Figure 2). Other notable changes observed were mild crypt
409 dilatation as well as a few samples showing crypt abscessation characterized by an accumulation
410 of sloughed-off epithelial cells and neutrophils. However, there was no effect of dietary treatment
411 on the inter-crypt area lymphocyte infiltration (P = 0.240) or crypt dilatation (P = 0.908). Dietary
412 treatment did not influence VH, CD, or VH:CD ratio in jejunum, ileum, or colon, as shown in
413 Table 11.
414 There were no differences in liver index among the dietary treatments (Supplementary Table 2).
415 Barrows had higher liver index compared to gilts (P = 0.008).
416

417 ## 3.5 SCFA and microbial community in the colon

418 There was no difference in the SCFA profile in the colon among the dietary treatments, except a
419 negative cubic tendency in the level of isovaleric acid (Table 12). The SCFA profile differed
420 between sexes. Barrows had higher levels of acetic acid (P = 0.035), propionic acid (P = 0.003),
421 and of total SCFA (P = 0.040), but lower levels of isobutyric acid (P = 0.045) and isovaleric acid
422 (P = 0.047) compared to gilts.
423

424 There was no difference in alpha diversity indices in the colon of piglets fed the different dietary
425 treatments (Figure 3a; N = 45, P = 0.355) as measured by the Shannon diversity index. Beta-
426 diversity was assessed by several distance methods (Bray-Curtis, unweighted and weighted
427 UniFrac). There were no differences in colon microbiota variance (beta dispersion) among the
428 dietary treatments with neither of the distance methods. The PERMANOVA test showed effect of

dietary treatment for the Bray-Curtis ($R^2 = 0.094$; $P = 0.029$) and unweighted UniFrac ($R^2 = 0.082$; $P = 0.041$) distances, but not for the weighted UniFrac distances ($R^2 = 0.084$; $P = 0.203$). However, when adjusted for multiple testing, the pairwise PERMANOVA tests gave no differences among any of the dietary treatments. The PCoA plot using Bray-Curtis distances (Panel b in Figure 3) shows no clear grouping of dietary treatments.

At the phylum level, the colon microbiota was dominated by Bacteroidota and Firmicutes (Figure 3, Panel c). The relative abundance of Bacteroidota differed among the dietary treatments ($P = 0.026$), whereas a tendency was found for the Firmicutes ($P = 0.055$). The Bacteroidota and Firmicutes were contributing 46.3% and 44.2% to the control group, 51.0% and 39.4% to the BSFL5 group, 50.0% and 42.1% to the BSFL10 group, and 48.1% and 38.9% to the BSFL20 group, respectively. There was also an effect of dietary treatment on Campilobacterota ($P =0.042$) and Thermoplasmatota ($P=0.002$), but the relative abundance of these two phyla were low in all groups (Campilobacterota: 1.0% in Control, 1.5% in BSFL5, 0.6% in BSFL10 and 0.5% in BSFL20; Thermoplasmatota: <0.01% in all groups).

Figure 4 shows the relative abundance of the top 10 most abundant genera in the colon. *Prevotella* was the most abundant genus, contributing 15.2%, 18.2%, 14.0% and 13.6% to the colon microbiome in pigs fed control, BSFL5, BSFL10, and BSFL20, respectively. An effect of dietary treatment was found for the relative abundance of *Lactobacillus*, where control pigs had higher relative abundance of *Lactobacillus* compared to pigs fed the BSFL20 diet. An effect of dietary treatment was also found for the *Rikenellaceae RC9* gut group, where higher relative abundance was found in the colon of pigs fed the BSFL10 diet compared to pigs fed the BSFL5 diet. No

covariation was found between the colonic SCFA concentrations and the microbiota composition (i.e. the overall ASV data; P = 0.366).

4. Discussion

The interest of using insects in animal feed is increasing (DiGiacomo and Leury, 2019; Varelas, 2019), and inclusion of BSFL have been investigated in diets for pigs (Spranghers et al., 2018), chicken (Józefiak et al., 2018), and fish (Nogales-Mérida et al., 2019; Weththasinghe et al., 2021), but to this date, there is limited information about the effect of high dietary inclusion levels. The present study demonstrated that up to 19% full-fat BSFL meal can be included as an alternative protein and energy source in pelleted diets for weaned piglets without affecting growth performance.

For a four-week period PW, pigs fed the control diet had the highest ADG, with a cubic effect of the BSFL inclusion level. Dietary inclusion of BSFL did not alter the ADFI or G:F. There was, however, a tendency for increased G:F with increased inclusion of BSFL the first 14 days PW. These results are partly consistent with Yu et al. (2020), which reported a linear improvement in both ADG and feed to gain ratio when feeding increasing BSFL inclusion from 0 to 4% in the two first weeks PW, whereas no differences were found for a four-week feeding period. Contrary, a study by Spranghers et al. (2018) showed no differences in performance when feeding up to 8% full-fat BSFL for 15 days, after weaning at 21 days of age.

Insects contain varying levels of chitin, depending on both types of insects and life stage (Finke, 2013). This polysaccharide is the major component of the insect's cuticle and creates a strong skeleton together with minerals and proteins. The exoskeleton of insects is different from crustaceans by having more amino acids strongly bound to the chitin (Finke, 2007; Andersen, 2010) and the degree of chitin deacetylation is dependent on the insect life stage (Smets et al., 2020). Inclusion of chito-oligosaccharide (derivates from chitosan) in a corn-soybean meal diet has been shown to reduce the incidences and score of diarrhea in piglets weaned at 16 days of age (Liu et al., 2008), and in piglets weaned at 21 days of age and challenged with *Escherichia coli* (Xiao et al., 2014). However, no difference in fecal score or fecal DM were found among the dietary treatments in this study and may be due to the complexity of the chitin in the insect cuticle and the low degree of acetalization and solubility compared to the chito-oligosaccharides (Guan et al., 2019). The increased fecal score in the first week PW for all treatments, shown in the supplementary figure, is in accordance with the critical period for PW diarrhea (Madec et al., 1998). The increase in fecal score in the last experimental week could be related to increased stress from sampling, as individual fecal samples for digestibility were collected daily in this period.

There is scarce information regarding the nutrient digestibility of insects and only a few studies including BSFL. Newton et al. (1977) found similar CTTAD of CP in five weeks old barrows fed 33% BSFL meal compared to the control diet. In the present study, there was reduced CTTAD of CP with increased inclusion of BSFL, but no difference in trypsin activity was found. A decline in the CTTAD of CP was also reported by Yu et al. (2020) when including low amounts of BSFL (<4%). Reduced CP digestibility with increased dietary inclusion of BSFL has also been reported for broiler chickens (Cullere et al., 2016), Atlantic salmon (Belghit et al., 2019; Weththasinghe et

al., 2021), and rainbow trout (Renna et al., 2017). The CP digestibility is found to negatively correlate with chitin content in BSFL, *in vitro* (Marono et al., 2015). Because of the nitrogen-rich chitin, the Kjeldahl-N method overestimates the digestible protein content of insects (Jonas-Levi et al., 2017). This is supported by the fact that the CIAD of total amino acids were not affected by dietary treatment. Janssen et al. (2017), suggested using a conversion factor of 5.6 g CP/g N to avoid the overestimation of protein content in BSFL.

Limited information is available about the amino acid digestibility of BSFL in pig diets. However, the amino acid digestibility of full-fat black soldier fly prepupae was recently investigated by Tan et al. (2020) and Crosbie et al. (2020). Tan et al. (2020) found the CIAD of amino acids in black soldier fly prepupae to be between 0.641 and 0.821, and the CISD to be between 0.767 and 1.177. Whereas, Crosbie et al. (2020) reported CIAD of amino acids to be between 0.616 and 0.874, and CISD values to be between 0.814 and 1.001 in full fat BSFL meal. During the diet optimization in the present study, the CISD for all amino acids in the BSFL meal were set to 0.83 because there was no reliable published CISD data at that time. All dietary treatments were formulated to have the same level of standardized ileal digestible lysine, but the CIAD of lysine decreased with increased inclusion of BSFL. Tan et al. (2020) reported a CISD of 0.776 for lysine in BSFL. The reduced CIAD of lysine in the present experiment, might therefore be explained by an overestimation of the CISD of lysine in the BSFL meal when formulating the experimental diets. A reduced apparent digestibility coefficient of lysine with increased inclusion of the same BSFL meal was also found in Atlantic salmon (Weththasinghe et al., 2021). However, Crosbie et al. (2020) reported the CISD of lysine to be 0.868, indicating more research is needed to define a CISD of lysine in BSFL. Maillard reactions are also known to reduce lysine availability in feed

519 (Fastinger and Mahan, 2006; Moughan and Rutherfurd, 2008), but there were no differences in
520 conditioner or pellet temperature during feed production (data not shown). On the other hand, the
521 CIAD of proline tended to increase with increased inclusion of BSFL. Proline is high in
522 endogenous losses, attributed to low reabsorption of mucin (Stein et al., 1999), but no information
523 about BSFL affecting endogenous losses was found in the literature. In general, the CIAD of amino
524 acids in the control diets was slightly lower compared with the control diet in a previous
525 experiment with this age pigs (Cruz et al., 2019). Especially the CIAD of tyrosine was in general
526 low and differed among the dietary treatments. The reported CISD for tyrosine in BSFL (0.824;
527 Tan et al. 2020) is only slightly lower than the coefficient used in the diet formulation (0.83). In
528 the method used for amino acid analysis, glucosamine, resulting from hydrolyzed insect chitin,
529 give a ninhydrin-positive peak close to the tyrosine peak in the chromatogram, which may cause
530 a higher uncertainty for accurate tyrosine analyses.
531
532 There was a linear increase in the CIAD and the CTTAD of CF with increased inclusion of BSFL,
533 whereas lipase activity measured in the oral part of jejunum showed no difference. The increased
534 digestibility of CF might be caused by a dilution of endogenous fat loss as explained by Jørgensen
535 et al. (1993). However, the authors believe that the total increase in the CTTAD of CF from 0.76
536 to 0.80 is only partly explained by this dilution factor due to the high level of fat in all diets. The
537 fat from BSFL amounted to be 19-69% of the total dietary fat and was affecting the fatty acid
538 profile of the diets. Saturated fatty acids increased with increased BSFL inclusion, whereas the
539 total amount of mono- and polyunsaturated fatty acids decreased. The increase in saturated fatty
540 acids was dominated by lauric acid (C12:0). The MCFA lauric acid is a major constituent of the
541 BSFLs fat. It is synthesized by the larvae, and therefore present at a high level independent of diet

(Spranghers et al., 2017; Ewald et al., 2020). Finke (2013) reported that lauric acid constituted 42% of the total fatty acids in the BSFL. The MCFAs are rapidly absorbed in the mucosa and, unlike long chain fatty acids, directly into the portal vein for transportation to the liver (Odle, 1997; Zentek et al., 2011). The differences in fatty acid composition among the diets are therefore believed to be the cause of the differences in the CTTAD of CF. Higher digestibility of fat was also reported with 8% (Spranghers et al., 2018) and 33% inclusion of BSFL (Newton et al., 1977). Contrary, Yu et al. (2020) found a lower CTTAD of CF when including 4% full-fat BSFL. Their reported CTTAD of CF was overall low (<70%). Different feed ingredients causing a different fatty acid profile of the diets, and low inclusion level of BSFL limiting the differences in fatty acid profiles between diets could explain the contradictory result by Yu et al. (2020).

Gilts showed higher CIAD and CTTAD of CP, CF, and ash, as well as for the CIAD of several amino acids, compared to barrows. Higher CTTAD of CP in gilts is previously reported by (Sheikh et al., 2017), including a nonsignificant increase in the CTTAD of ether extract. The use of wood shavings as bedding material also increased the numerical CTTAD of all nutrients except for aNDF. This is probably a result of pigs eating the wood shavings. Dietary fiber is known to decrease the CTTAD of nutrients (Wilfart et al., 2007), but when not included in the dietary composition it can disturb digestibility calculations.

The MCFA can directly supply the enterocytes with energy and thereby improve morphological changes in periods with nutrient deficiency such as weaning (Zentek et al., 2011). It is well known that weaning causes villus atrophy. However, both in this study and the study by Spranghers et al. (2018), no effect of BSFL inclusion was found on intestinal morphometry. The effect of MCFA

565 on morphology could be more evident the first days PW as the VH in the small intestine seems to
566 recover within two weeks PW (Håkenåsen et al., 2020). Biasato et al. (2019) also reported no
567 difference in intestinal morphometric indices after feeding pigs diets with up to 10% defatted
568 BSFL. In accordance with our results, they also observed greater morphometric indices in the
569 jejunum than the ileum.
570
571 The gut health was mildly suboptimal due to the observation of inflammation and edema in the
572 jejunum and colon of some piglets. However, except for the jejunal enterocyte vacuolization, the
573 general histopathological findings did not appear to be associated with the dietary treatments. The
574 results are consistent with Biasato et al. (2019) which replaced soybean meal with defatted BSFL,
575 concluding that up to 10% BSFL inclusion does not negatively affect gut morphology or
576 histological features. The mild morphological changes (submucosal and lamina propria edema) in
577 some of the pigs could be related to the edema disease outbreak. The mild to moderate enterocyte
578 vacuolization at the folder tips of the jejunum could be related to an accumulation of lipids (or
579 other nutrients) in the enterocytes. Randazzo et al. (2021) observed increased enterocyte
580 vacuolization in the medium intestine of fish fed diets with both *Hermetia illucens* prepupae meal
581 and vegetable-protein mixture and demonstrated by Fourier Transform Infrared Imaging (FTIR)
582 analysis that this was due to improved lipid absorption. No special staining or FTIR analysis for
583 further characterization of the vacuoles were conducted in the present study, thus further
584 investigation is needed in this regard.
585
586 The microbiota of the gut is involved in the nutritional, physiological, and immunological
587 functions of the pig (Fouhse et al., 2016). Variation in the diet composition is one of the most

588 important factors affecting the GIT microbial ecosystem of the pig (Rist et al., 2013). The
589 microbial community in the colon was dominated by Bacteroidota and Firmicutes, also reported
590 by Yu et al. (2019). Yu and coworkers found no differences in phyla abundances when including
591 4 or 8% full-fat BSFL in diets for finishing pigs. By contrast, the relative abundance of
592 Bacteroidota, Firmicutes, Campilobacterota, and Thermoplasmatota differed among the dietary
593 treatments in the present study, but there was no clear dose-effect of the dietary BSFL inclusion.
594 However, the highest relative abundance of Firmicutes and lowest relative abundance of
595 Bacteroidota was found in pigs fed the control diet. In accordance with Yu et al. (2019), no
596 significant differences in Shannon indices were observed.
597
598 *Prevotella* was the most abundant genus in the colon of the pigs regardless of dietary treatment.
599 This is in correspondence with several authors reporting *Prevotella* to be the most abundant genus
600 in the fecal microbiome in the PW period (Isaacson and Kim, 2012; Guevarra et al., 2018; Wang
601 et al., 2019). *Lactobacillus* was more abundant in the colon of the control piglets. This result is
602 contrary to what recently was reported by Yu et al. (2019), where inclusion of 4% BSFL increased
603 the abundance of *Lactobacillus*. In the study by Yu et al. (2019), finishing pigs were fed corn-
604 based diets contrary to our PW pigs, which were fed wheat and barley-based diets. Also, when
605 they increased the inclusion to 8% BSFL, Yu et al. (2019) did not find the same beneficial effects
606 on the colonic microbiota as when feeding the 4% inclusion of BSFL. *Lactobacillus* is a genus of
607 gram-positive lactic acid-producing bacteria in general considered favorable in the gut
608 microbiome. It is known that lauric acid, abundantly found in BSFL fat, has anti-microbial effects,
609 especially against gram-positive bacteria (Zentek et al., 2011). Spranghers et al. (2018), observed
610 inhibition of the growth of lactobacilli in an *in vitro* study with BSFL fat but did not observe

differences *in vivo* in the small intestine. They also observed that a high amount of the lauric acid was absorbed already in the stomach and therefore had little opportunity to modulate the colon microbiome. In rainbow trout, it is shown that the BSFL life stage and lipid content are important factors influencing the gut microbiome (Huyben et al., 2019).

In the present experiment, the content of several protein and fat ingredients, including soybean meal, soy protein concentrate, fish meal, rapeseed oil, and saturated vegetable fat, was reduced at the expense of increased BSFL inclusion in the diets. It is therefore not possible to differentiate if the observed chances are due to the BSFL inclusion or the reduction of other ingredients. It is also important to mention the treatment with antibiotics of all pigs in the second week of the experiment. The effect of the antibiotic on the gut microbiota is antibiotic specific (Fouhse et al., 2016), and it is therefore difficult to know what impact the antibiotic treatment of the pig in this experiment had on their gut microbiota. Indeed, the antibiotic treatment could have affected microbiota results and be the reason for the small differences in the colon microbiome among the dietary treatments. Because of the antibiotic treatment in the second experimental week, it might be that the feeding period was too short to cause significant changes in the gut microbiome. Also, therapeutic doses of antibiotics might suppress the innate immune defenses and contribute to increased disease susceptibility (Fouhse et al., 2016). Almost half of the pigs, independent of dietary treatment, had reddening of the colonic mucosa, indicating some inflammation in the colonic intestinal wall. However, the overall Shannon diversity indices reported were similar to what was reported by Yu et al. (2019) in finishing pigs.

5. Conclusion

To conclude, up to 19.06% of full-fat BSFL meal could be included in a balanced diet for PW pigs with minor effects on performance results. Increased inclusion of BSFL decreased the CTTAD of CP and increased CTTAD of CF but did not affect the general gut function, as assessed by enzyme activity and morphometry. There was a mild to moderate enterocyte vacuolization at the jejunal folder tips in piglets fed insect meal. Some changes in the colon microbiota composition were observed, such as decreased relative abundance of *Lactobacillus* with dietary inclusion of BSFL. However, there are limited information and contradictive results on the effect of BSFL on the gut microbiome in pigs, thus further investigations are needed.

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Figure legends

Figure 1 Main findings from the visual inspection of intestinal tissue prior to the collection of tissue for histology. **Panel a:** Normal jejunum mucosa. **Panel b:** Jejunal mucosal with low muscle tone and complete loss of mucosal folds. **Panel c:** Normal appearance of ileum mucosa as observed for most of the samples. **Panel d**: Normal and healthy appearance of the colon. **Panel e:** Colon mucosa with mild mucosal reddening.

Figure 2 Representative images of the morphological appearance of the colon tissue showing the histopathological changes of **a**: lymphocytic and plasma cell infiltration of the inter-crypt area (orange arrow), and **b**: crypt abscessation (black arrow) characterized by an accumulation of neutrophils in the crypt lumen and surrounded by crypt epithelial cells that have reduced numbers (black arrowhead) of goblet cells compared to healthy crypt epithelium (blue arrowhead). Scale bars in both images represent a distance of 100 μm.

Figure 3 Colon microbiota diversity in pigs fed an increased amount of BSFL**. Panel a:** Boxplot of Shannon diversity indices. The median value is represented as the lines inside the boxes. **Panel b:** Principal coordinate analysis plot with Bray-Curtis distances of colon microbiota colored by diet. **Panel c:** Average relative abundance of the microbial populations at the phylum level for each of the dietary treatments.

Figure 4 Relative abundance of top 10 abundant genera. Brackets denote significant differences among dietary treatments.

Supplementary material

Supplementary Figure 1 Development of the overall fecal score during the experimental period.

Supplementary Table 1 Nutrient composition of feed ingredients.

Supplementary Table 2 pH, enzyme activity and liver index in post-weaning pigs (average body weight 25 kg) fed increasing levels (5, 10, and 20%) of black soldier fly larvae meal (BSFL).

Tables

Table 1 Dietary composition of experimental diets, calculated total crude protein (CP) content in diets, and calculated CP and crude fat (CF) from black soldier fly larvae (BSFL) meal.

	Dietary treatments			
Ingredients, g/kg as fed[a]	Control	BSFL5	BSFL10	BSFL20
Wheat	507.7	502.0	496.6	485.1
Barley	200.0	200.0	200.0	200.0
Oats	50.0	50.0	50.0	50.0
BSFL meal[b]	—	47.6	95.2	190.6
Soybean meal[c]	70.8	59.7	48.2	25.5
Soy protein concentrate[d]	36.1	27.1	18.1	—
Fish meal[e]	34.1	25.5	17.1	—
Rapeseed oil	32.8	25.1	17.4	1.3
Saturated vegetable fat[f]	11.0	8.1	5.1	—
Rapeseed meal[g]	10.0	10.0	10.0	10.0
Monocalcium phosphate	12.9	12.0	11.2	9.5
Limestone	8.2	6.7	5.1	2.1
Sodium chloride	5.3	5.4	5.5	5.8
Selenium premix	0.9	0.9	0.8	0.8
Iron(II)fumarate	0.4	0.4	0.4	0.4
Micro-mineral premix[h]	2.0	2.0	2.0	2.0
Vitamins[i]	3.0	3.0	3.0	3.0
L-Lysine	6.8	6.8	6.9	6.9
L-Methionine	2.4	2.5	2.6	2.8
L-Threonine	2.8	2.9	2.9	3.0
L-Valine	1.1	0.8	0.5	0.0
L-Tryptophan	0.9	0.8	0.7	0.6
Betaine	0.7	0.7	0.7	0.7
Yttrium(III)oxide	0.1	0.1	0.1	0.1
Ratio CP from BSFL (% of total CP)	0	10	20	39
Ratio CF from BSFL (% of total CF)	0	19	39	69

[a] Chemical composition of main ingredients are provided in Supplementary Table 1.
[b] HiProMine S.A., Poznanska Str, Poland
[c] Non-GMO soybean meal, Denofa AS, Fredrikstad, Norway.
[d] AX3 Gastric, TripleA a/s, Hornsyld, Denmark.
[e] Nordsildmel AS, Egersund, Norway.
[f] AkoFeed Gigant 60, AAK AB, Malmö, Sweden.
[g] Expeller-pressed rapeseed cake, Mestilla, UAB, Klaipeda Lithuania.
[h] "Mikro-Svin"; provided per kilogram of diet: 120 mg Zn (ZnO); 120 mg Fe, 26 mg Cu, 13.2 mg S ($FeSO_4$, $CuSO_4$); 60 mg Mn (MnO); 3.4 mg Mg ($CaMg(CO_3)_2$); 0.6 mg I ($Ca(IO_3)_2$).

[i] Provided per kilogram of diet: 0.7 g Vitamin A; 1.2 g Vitamin E v5; 0.8 g Vitamin ADKB mix; 0.3 g Vitamin C (Stay C 35%).

Table 2 Analyzed and calculated chemical composition of dietary treatments.

	Dietary treatments[a]			
Nutrients, g/kg DM	Control	BSFL5	BSFL10	BSFL20
Dry matter, g/kg	890.3	888.9	887.7	893.0
Ash	54.3	52.3	52.5	52.9
Crude protein	196.8	196.1	199.8	206.8
Starch	512.5	523.8	504.1	488.0
Crude fat	69.3	79.7	78.6	89.1
aNDF	124.1	122.9	118.3	131.0
ADF	52.9	52.0	50.2	53.8
Gross energy (MJ/kg DM)	19.5	19.6	19.8	19.9
Phosphorus	7.4	7.3	7.6	7.0
Calculated net energy (MJ/kg)	10.47	10.47	10.47	10.47
Amino acids, calculated SID[b] (g/kg)				
Lysine	12.5	12.5	12.5	12.5
Methionine + Cysteine	7.4	7.4	7.4	7.4
Threonine	7.9	7.9	7.9	7.9
Tryptophan	2.8	2.8	2.8	2.8
Valine	8.4	8.4	8.4	8.5

[a] BSFL: black soldier fly larvae (dietary inclusion level 5, 10, and 20%)

[b] SID: Standardized ileal digestibility

Table 3 Analyzed amino acid (AA) composition of dietary treatments.

		Dietary treatments[a]			
Indispensable AA, g/kg[b]	BSFL meal	Control	BSFL5	BSFL10	BSFL20
Arginine	16.7	8.6	8.5	8.1	7.8
Histidine	9.0	3.7	3.8	3.9	4.3
Isoleucine	16.4	5.7	5.8	5.7	6.2
Leucine	30.4	10.6	10.5	10.4	10.8
Lysine	21.9	11.8	12.5	11.7	12.7
Methionine	6.5	4.0	4.3	4.1	4.6
Phenylalanine	15.0	6.7	6.5	6.4	7.0
Threonine	15.1	7.1	7.6	7.2	7.9
Tryptophan		2.5	2.1	2.5	2.5
Valine	21.5	7.0	7.1	7.0	7.5
Dispensable AA, g/kg					
Alanine	27.8	5.7	6.2	6.5	7.6
Aspartic acid	33.2	11.8	12.0	11.6	11.8
Cysteine	2.7	2.3	2.3	2.2	2.1
Glutamic acid	43.8	34.9	33.2	33.2	33.6
Glycine	18.2	5.7	5.8	5.8	6.3
Proline	20.6	10.4	10.3	10.7	11.7
Serine	14.2	6.6	6.5	6.4	6.5
Tyrosine	20.3	3.0	3.2	3.9	5.7
Total amino acids	333.4	145.6	146.1	144.7	154.1

[a] BSFL: black soldier fly larvae (dietary inclusion level 5, 10, and 20%)
[b] Determined using water-corrected molecular weights

1013 **Table 4** Analyzed fatty acid composition of dietary treatments, and calculated sum of saturated-,
1014 monounsaturated-, and polyunsaturated fatty acids.

		Dietary treatments[a]			
Fatty acids, g/kg DM	BSFL meal	Control	BSFL5	BSFL10	BSFL20
C12:0	95.82	0.40	6.11	11.7	23.1
C14:0	19.29	0.42	1.35	2.33	4.25
C15:0	0.44	0.04	0.06	0.07	0.09
C16:0	31.80	13.3	12.4	11.4	9.96
C17:0	0.60	0.07	0.08	0.10	0.13
C18:0	7.05	4.31	3.49	2.76	1.54
C20:0	0.29	0.24	0.20	0.15	0.09
C21:0	1.73	0.01	0.09	0.28	0.32
C22:0	0.09	0.14	0.11	0.08	0.04
C23:0	-	0.02	0.01	0.01	-
C24:0	-	0.05	0.04	0.03	0.02
Sum saturated fatty acids	157.11	19.02	23.95	28.89	39.57
C14:1	0.36	-	0.02	0.04	0.08
C16:1	5.18	0.40	0.58	0.76	1.13
C18:1n9t	0.10	0.46	0.37	0.21	0.04
C18:1n9c	33.49	25.9	22.8	19.0	12.2
C20:1	0.22	0.89	0.73	0.55	0.22
C21:1n9	-	0.05	0.05	0.04	0.02
C24:1	-	0.11	0.08	0.04	0.03
Sum monounsaturated fatty acids	39.35	27.81	24.63	20.64	13.72
C18:2n6c	40.82	21.1	22.0	21.7	21.1
C18:3n3	5.31	4.56	4.15	3.56	2.39
C18:3n6	0.05	0.13	0.11	0.08	0.02
C20:2	0.12	0.13	0.11	0.09	0.05
C20:4n6	-	0.02	0.02	0.01	-
C20:5n3	-	0.30	0.23	0.16	-
C22:2	-	0.04	0.02	0.02	0.02
C22:5n3	-	0.03	0.02	0.01	-
C22:6n3	-	0.36	0.30	0.20	-
Sum polyunsaturated fatty acids	43.30	26.67	26.96	25.83	23.58

1015 [a] BSFL: black soldier fly larvae (dietary inclusion level 5, 10, and 20%)

1016 **Table 5** Growth performance parameters for post-weaning (PW) pigs (N = 20) fed increasing levels (5, 10,
1017 and 20%) of black soldier fly larvae meal (BSFL).

		Dietary treatments					P-value		
Day PW	**Items**[a]	Control	BSFL5	BSFL10	BSFL20	SEM[b]	Linear	Quadratic	Cubic
	Initial BW	10.7	10.6	10.6	10.6	0.1	0.842	0.893	0.992
	Final BW	25.7	24.0	25.2	24.9	0.3	0.662	0.317	0.056
	ADFI	485	448	459	464	9	0.633	0.324	0.430
0-14	ADG	432	392	425	427	7	0.682	0.267	0.060
	G:F	0.87	0.88	0.93	0.92	0.01	0.052	0.303	0.209
	ADFI	943	867	918	917	15	0.939	0.348	0.152
14-27	ADG	683	608	661	636	15	0.502	0.470	0.085
	G:F	0.72	0.70	0.72	0.69	0.01	0.411	0.880	0.332
	ADFI	712	650	680	682	11	0.679	0.233	0.162
0-27	ADG	552	496	538	528	9	0.720	0.279	0.031
	G:F	0.79	0.76	0.79	0.77	0.01	0.949	0.776	0.187

1018 [a] BW: body weight, kg; ADG: average daily gain, g; ADFI: average daily feed intake, g; G:F: gain to feed ratio
1019 [b] SEM: standard error of mean
1020

1021 **Table 6** Fecal dry matter (DM) and fecal score in post-weaning pigs (N = 20) fed increasing levels (5, 10,
1022 and 20%) of black soldier fly larvae meal (BSFL). A higher fecal score indicates more watery feces.

	Dietary treatments					P-value		
Fecal DM, %	Control	BSFL5	BSFL10	BSFL20	SEM[a]	Linear	Quadratic	Cubic
Day 7	26.6	25.7	25.3	24.3	0.7	0.271	0.917	0.898
Day 14	27.8	26.1	26.9	26.3	0.3	0.190	0.385	0.144
Day 21	26.3	26.2	26.1	24.7	0.4	0.128	0.520	0.937
Day 27	26.7	26.3	27.3	26.7	0.4	0.879	0.805	0.489
Total average	26.8	26.1	26.4	25.5	0.3	0.182	0.987	0.482
Fecal Score								
Week 1	1.82	1.87	1.87	1.84	0.06	0.954	0.777	0.932
Week 2	1.83	1.99	1.88	1.91	0.06	0.861	0.656	0.395
Week 3	1.73	1.78	1.56	1.72	0.06	0.801	0.445	0.269
Week 4	1.86	2.00	1.86	1.95	0.05	0.785	0.947	0.312
Overall period	1.82	1.92	1.80	1.86	0.05	0.960	0.990	0.344

1023 [a] SEM: standard error of mean
1024

Table 7 Coefficients of ileal apparent digestibility (CIAD) in post-weaning pigs (average body weight 25.5 kg) fed increasing levels (5, 10, and 20%) of black soldier fly meal (BSFL).

		Dietary treatments					P-value		
CIAD	N	Control	BSFL5	BSFL10	BSFL20	SEM[a]	Linear	Quadratic	Cubic
Dry matter	40	0.711	0.674	0.680	0.680	0.010	0.375	0.294	0.470
Crude protein	40	0.697	0.659	0.666	0.676	0.014	0.624	0.303	0.543
Starch	40	0.974	0.949	0.953	0.958	0.005	0.448	0.131	0.361
Crude fat	40	0.817	0.817	0.848	0.868	0.010	0.043	0.894	0.445
Ash	37	0.309	0.281	0.266	0.407	0.020	0.069	0.031	0.793

[a] SEM: standard error of mean

Table 8 Coefficients of ileal apparent digestibility (CIAD) for amino acids (AA) in post-weaning pigs (average body weight 25.5 kg) fed increasing levels (5, 10, and 20%) of black soldier fly larvae meal (BSFL).

		Dietary treatments					P-value		
CIAD of AA[a]	N	Control	BSFL5	BSFL10	BSFL20	SEM[b]	Linear	Quadratic	Cubic
Indispensable AA									
Arginine	40	0.811	0.802	0.784	0.782	0.008	0.076	0.560	0.701
Histidine	39	0.783	0.763	0.741	0.757	0.006	0.073	0.030	0.581
Isoleucine	40	0.778	0.757	0.738	0.775	0.008	0.835	0.038	0.720
Leucine	40	0.790	0.768	0.752	0.781	0.008	0.671	0.047	0.843
Lysine	40	0.865	0.864	0.841	0.842	0.005	0.020	0.297	0.231
Methionine	40	0.873	0.876	0.855	0.884	0.005	0.494	0.053	0.080
Phenylalanine	40	0.795	0.760	0.736	0.778	0.008	0.445	0.005	0.740
Threonine	40	0.758	0.752	0.738	0.764	0.009	0.922	0.241	0.705
Valine	40	0.792	0.763	0.746	0.772	0.009	0.409	0.055	0.941
Dispensable AA									
Alanine	40	0.692	0.673	0.683	0.740	0.012	0.105	0.119	0.745
Aspartic acid	40	0.675	0.655	0.667	0.699	0.013	0.466	0.278	0.646
Cysteine	40	0.637	0.564	0.604	0.600	0.019	0.635	0.351	0.229
Glutamic acid	40	0.811	0.770	0.797	0.813	0.011	0.719	0.243	0.237
Glycine	40	0.425	0.397	0.432	0.531	0.032	0.220	0.402	0.747
Proline	39	0.635	0.667	0.732	0.742	0.022	0.069	0.478	0.595
Serine	40	0.735	0.709	0.706	0.715	0.010	0.486	0.241	0.753
Tyrosine	40	0.576	0.503	0.411	0.481	0.017	0.036	0.005	0.321
Total amino acids	40	0.750	0.733	0.734	0.756	0.010	0.795	0.279	0.817

[a] Determined using water-corrected molecular weights

[b] SEM: standard error of mean

Table 9 Coefficients of total tract apparent digestibility (CTTAD) in post-weaning pigs (average body weight 25 kg) fed increasing levels (5, 10, and 20%) of black soldier fly larvae meal (BSFL).

		Dietary treatments					P-value		
CTTAD	N	Control	BSFL5	BSFL10	BSFL20	SEM[a]	Linear	Quadratic	Cubic
Dry matter	79	0.834	0.828	0.830	0.827	0.002	0.292	0.682	0.490
Crude protein	79	0.796	0.784	0.775	0.773	0.004	0.011	0.184	0.886
Starch[b]	79	0.997	0.997	0.998	0.998	<0.001	0.051	0.339	0.065
Crude fat	79	0.757	0.780	0.794	0.800	0.004	<0.001	0.058	0.985
ADF	78	0.303	0.279	0.284	0.292	0.011	0.800	0.395	0.686
aNDF	77	0.399	0.369	0.375	0.394	0.007	0.954	0.109	0.507
Phosphorus	79	0.513	0.515	0.553	0.564	0.006	<0.001	0.574	0.092
Ash	79	0.587	0.574	0.576	0.612	0.005	0.014	0.015	0.911
Energy	79	0.826	0.820	0.823	0.818	0.002	0.255	0.794	0.416

[a] SEM: standard error of mean
[b] 26 feces samples had starch levels below lower detection limit

Table 10 Summary of the main intestinal macroscopic findings in post-weaning pigs fed increasing levels (5, 10, and 20%) of black soldier fly larvae meal (BSFL).

	Dietary treatments				
Macroscopic findings[a]	Control	BSFL5	BSFL10	BSFL20	P-value
Reddening of the jejunal mucosa	3	3	1	2	0.702
Jejunum with low muscle tone and no mucosal folding	2	4	2	1	0.523
Reddening of the ileal mucosa		1	2	1	0.893
Reddening of the colonic mucosa	5	6	4	6	0.864

[a] Numbers represent total samples observed with specific changes (N = 47).

1051 **Table 11** Intestinal morphometry (N = 45) in post-weaning pigs fed increasing levels (5, 10, and 20%) of
1052 black soldier fly larvae meal (BSFL).

	Dietary treatments					P-value		
Morphometry[a], µm	Control	BSFL5	BSFL10	BSFL20	SEM[b]	Linear	Quadratic	Cubic
Jejunum								
VH	454	457	472	465	13	0.747	0.747	0.757
CD	413	389	368	398	12	0.724	0.238	0.861
VH:CD ratio	1.23	1.25	1.37	1.30	0.06	0.628	0.620	0.681
Ileum								
VH	232	233	250	247	7	0.316	0.680	0.544
CD	134	148	150	137	4	0.909	0.136	0.775
VH:CD ratio	1.92	1.64	1.75	1.96	0.6	0.493	0.118	0.381
Colon								
CD	486	479	480	482	9	0.907	0.754	0.966

1053 [a] VH: villus heigh, CD: crypt depth
1054 [b] SEM: standard error of mean
1055

1056 **Table 12** Short chain fatty acid (SCFA) and ammonia concentration in colon content in post-weaning
1057 pigs fed increasing levels (5, 10, and 20%) of black soldier fly larvae meal (BSFL).

		Dietary treatments					P-value		
SCFA, µmol/g	N	Control	BSFL5	BSFL10	BSFL20	SEM[a]	Linear	Quadratic	Cubic
Acetic acid	47	56.5	51.9	52.7	52.1	1.63	0.428	0.492	0.565
Butyric acid	47	13.5	10.4	12.4	12.6	0.62	0.926	0.165	0.110
Isobutyric acid	47	0.87	0.69	0.87	0.78	0.06	0.774	0.807	0.218
Isovaleric acid	47	1.05	0.79	1.11	0.92	0.08	0.793	0.979	0.093
Propionic acid	47	25.6	21.8	24.2	24.5	0.84	0.941	0.367	0.168
Valeric acid	46	1.96	1.75	1.70	1.77	0.11	0.533	0.405	0.936
Total SCFA	47	99.8	87.4	93.0	92.3	2.68	0.562	0.310	0.232
Ammonia, mM	47	3.00	2.72	2.87	2.70	0.08	0.309	0.724	0.344

1058 [a] SEM: standard error of mean
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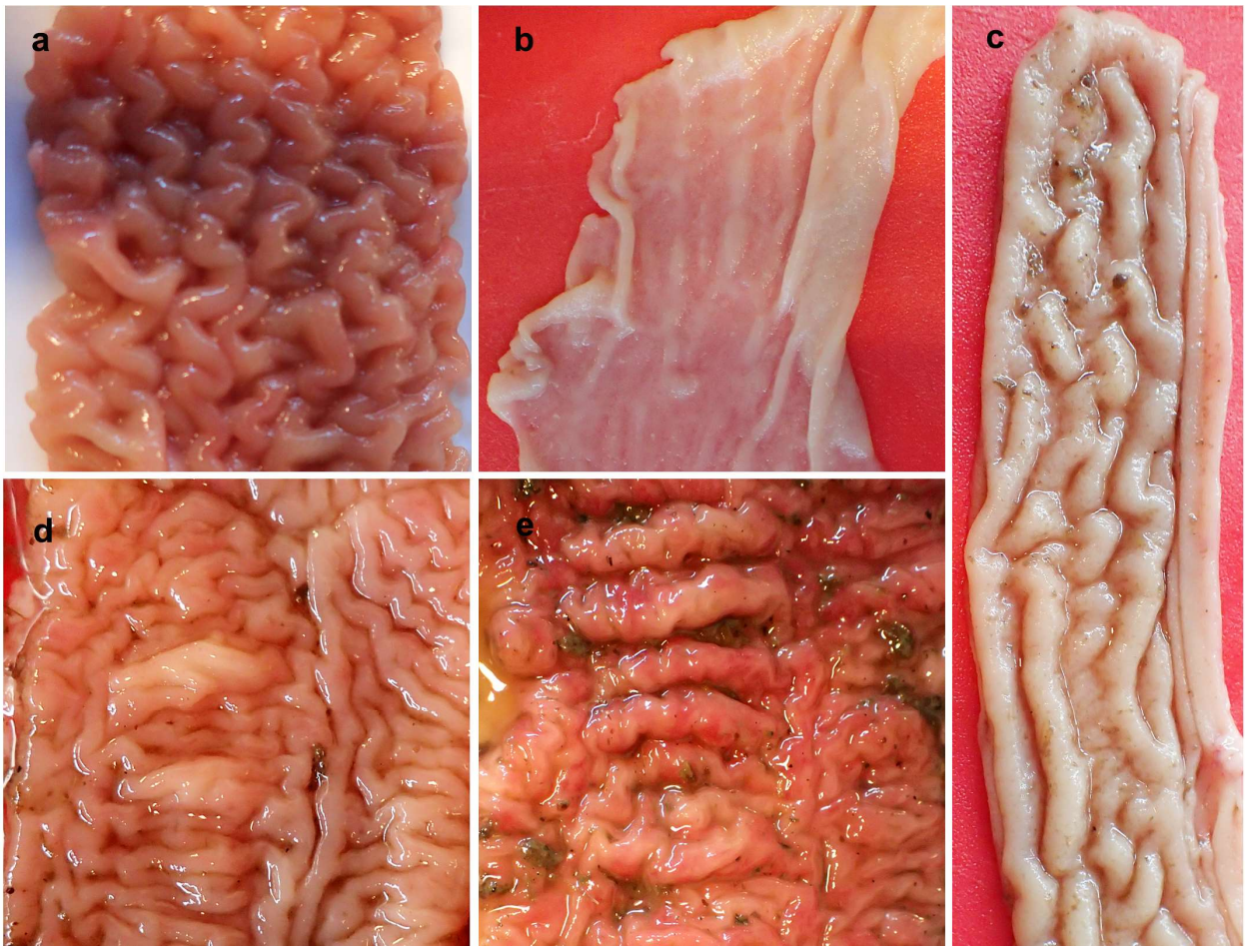
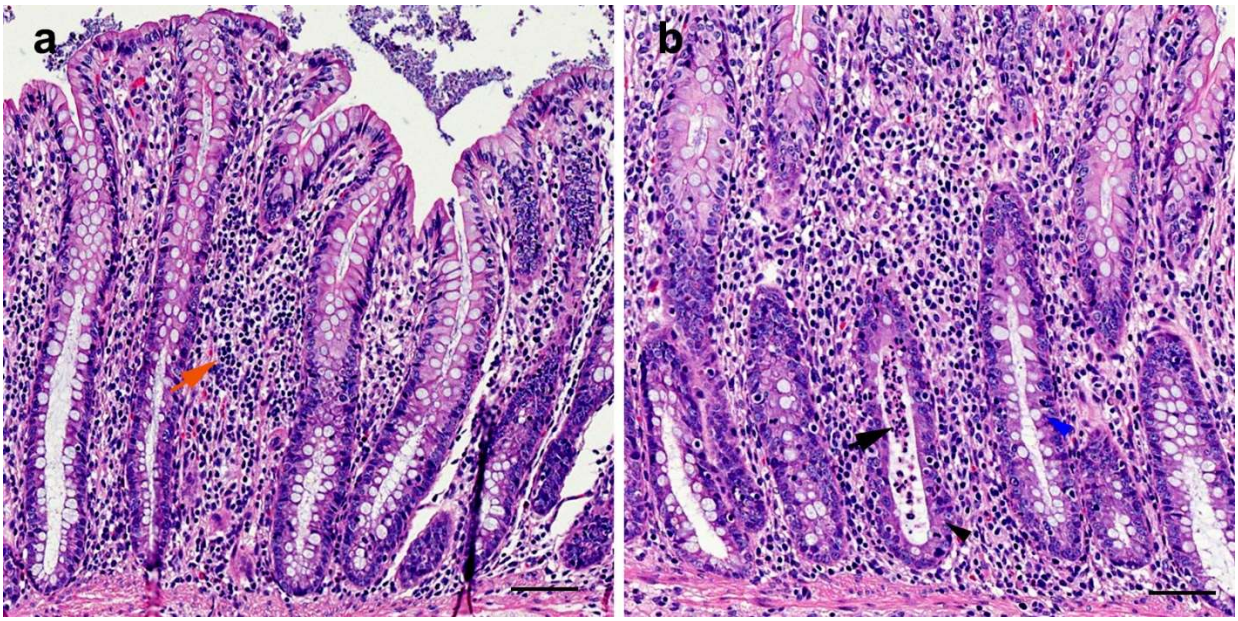


Figure 1.

1073



1074

1075 Figure 2.

1076

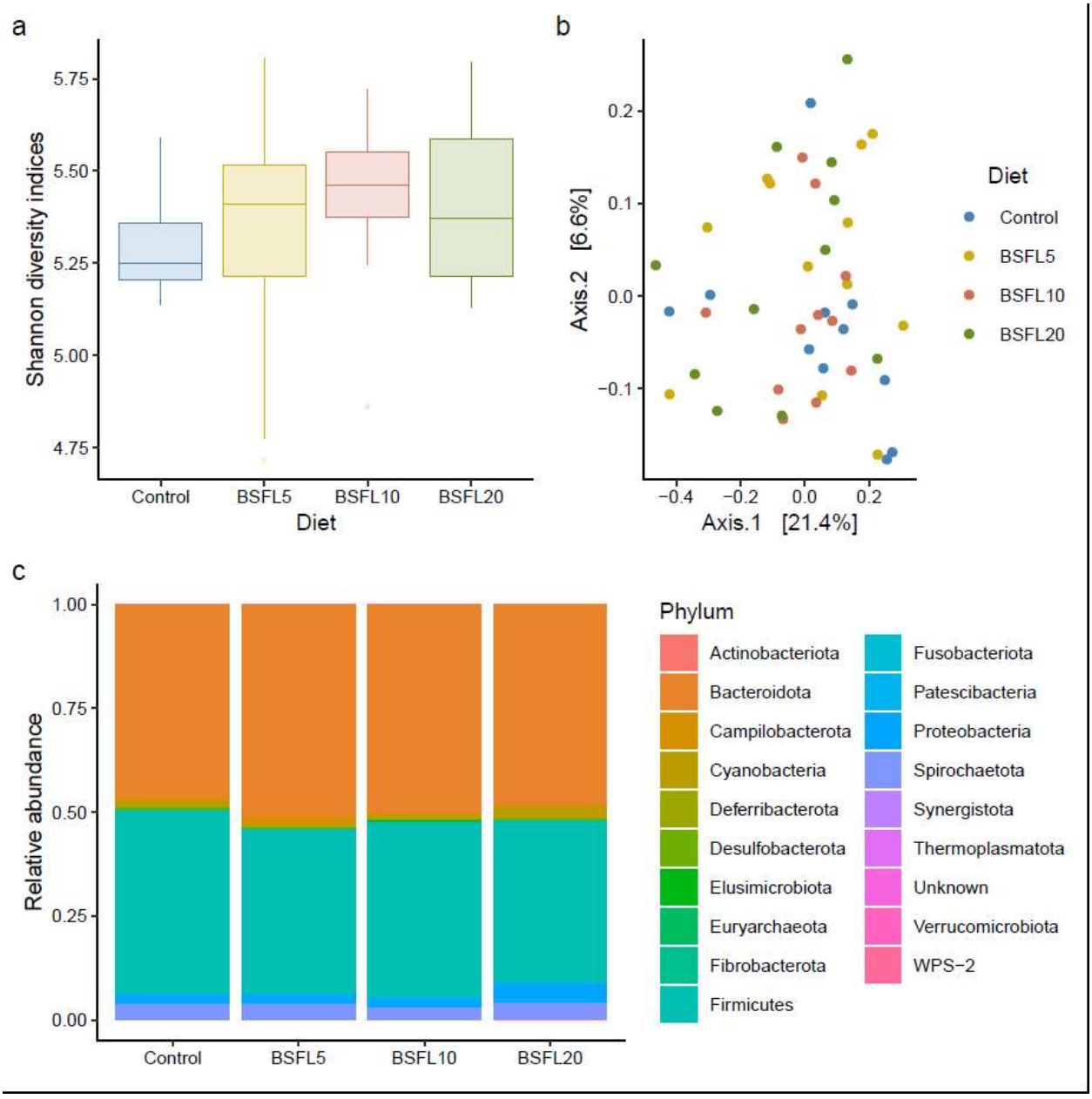

a
Shannon diversity indices
5.75
5.50
5.25
5.00
4.75
Control
BSFL5
BSFL10
BSFL20
Diet
b
Axis.2 [6.6%]
0.2
0.1
0.0
−0.1
−0.4
−0.2
0.0
0.2
Axis.1 [21.4%]
Diet
Control
BSFL5
BSFL10
BSFL20
c
Relative abundance
1.00
0.75
0.50
0.25
0.00
Control
BSFL5
BSFL10
BSFL20
Phylum
Actinobacteriota
Bacteroidota
Campilobacterota
Cyanobacteria
Deferribacterota
Desulfobacterota
Elusimicrobiota
Euryarchaeota
Fibrobacterota
Firmicutes
Fusobacteriota
Patescibacteria
Proteobacteria
Spirochaetota
Synergistota
Thermoplasmatota
Unknown
Verrucomicrobiota
WPS−2

Figure 3.

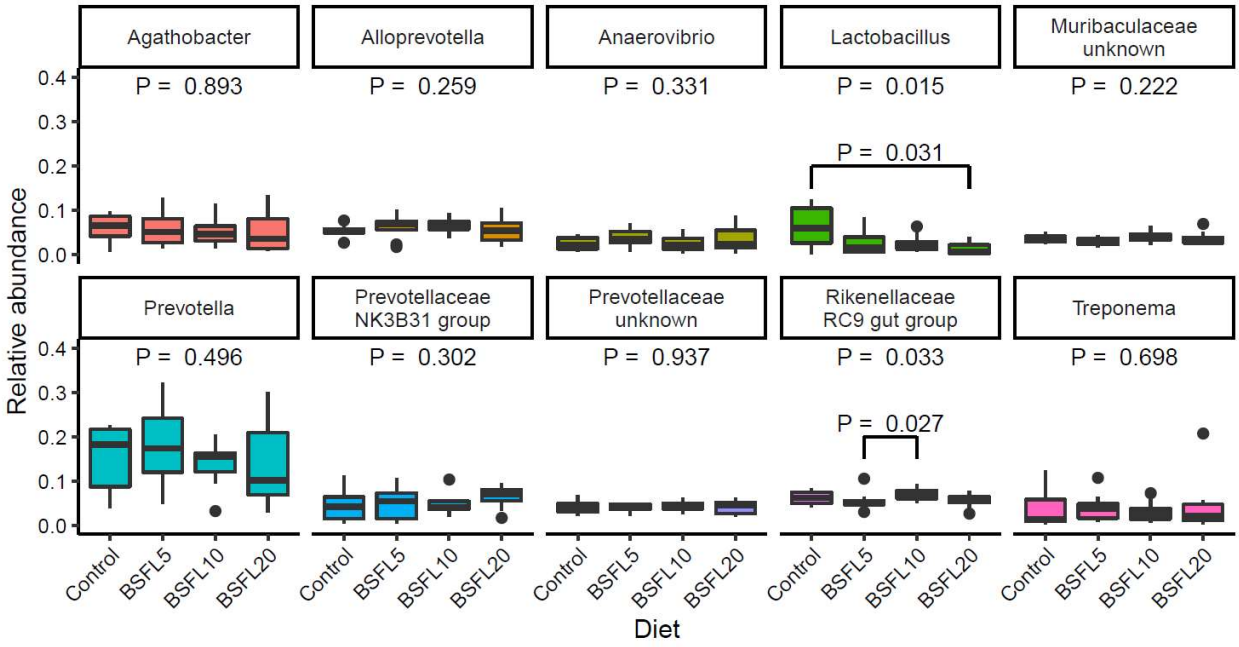
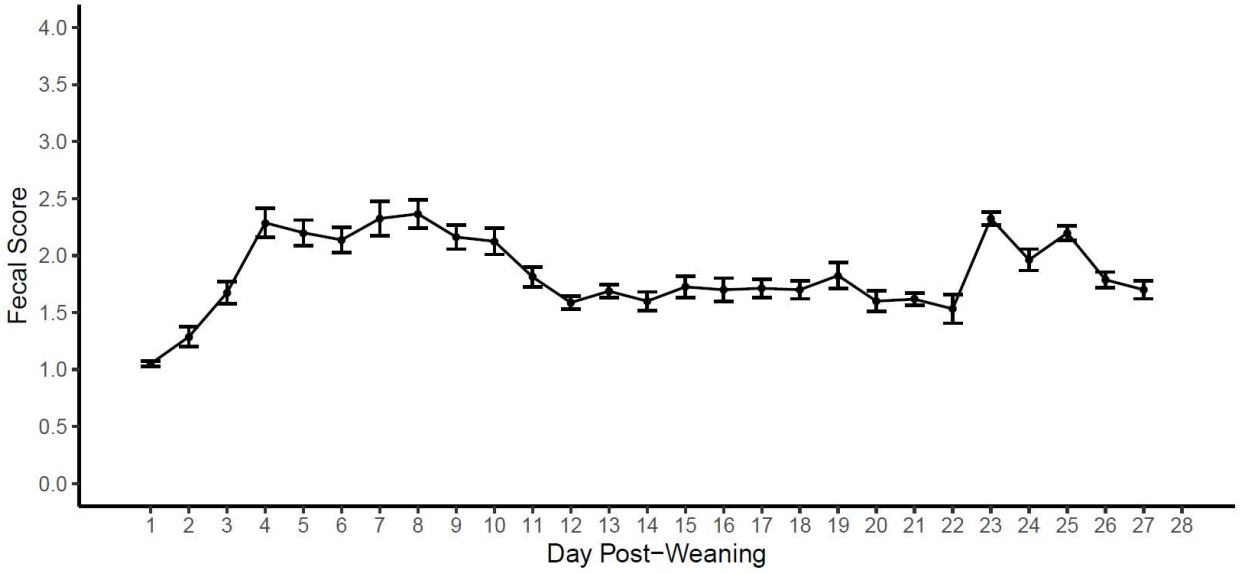

Agathobacter
Alloprevotella
Anaerovibrio
Lactobacillus
Muribaculaceae unknown
P = 0.893
P = 0.259
P = 0.331
P = 0.015
P = 0.222
P = 0.031
Prevotella
Prevotellaceae NK3B31 group
Prevotellaceae unknown
Rikenellaceae RC9 gut group
Treponema
P = 0.496
P = 0.302
P = 0.937
P = 0.033
P = 0.698
P = 0.027
Relative abundance
0.0
0.1
0.2
0.3
0.4
Control
BSFL5
BSFL10
BSFL20
Diet

Figure 4.

Supplementary material



Supplementary Figure 1

Supplementary Table 1 Nutrient composition (g/kg) of feed ingredients.

Ingredient	Dry matter	Crude protein	Starch	Crude fat	ADF	aNDF	Ash	Chitin	Energy (MJ/kg)
Wheat	867	124	488	14	45	116	14		16
Barley	858	92	564	11	82	206	17		16
Oats	858	88	408	44	150	270	23		17
BSFL meal[a]	905	380	-	289	-	-	84	7.2	-
Soybean meal	876	426	-	11	128	156	53		17
Fish meal	920	679	-	101	-	-	147		19
Rapeseed meal	928	342	-	100	179	223	53		20

[a] **Ca: 17.4 g/kg, Mg: 2.4 g/kg, total P: 7.8 g/kg.**

Supplementary Table 2 pH, enzyme activities and liver index in post-weaning pigs (average body weight 25 kg) fed increasing levels (5, 10, and 20%) of black soldier fly larvae meal (BSFL).

		Dietary treatments					P-value		
	N	Control	BSFL5	BSFL10	BSFL20	SEM[a]	Linear	Quadratic	Cubic
pH stomach	47	3.8	3.9	3.7	3.6	0.1	0.579	0.751	0.598
pH jejunum	47	5.4	5.3	5.3	5.2	0.1	0.479	0.971	0.649
Trypsin jejunum, mU/µg protein	42	1.04	0.90	1.06	0.68	0.14	0.457	0.584	0.767
Lipase jejunum, mU/mg protein	45	0.07	0.07	0.08	0.06	0.01	0.839	0.810	0.839
Liver index	44	2.65	2.58	2.64	2.74	0.04	0.127	0.456	0.624

[a] SEM: standard error of mean

*5.1. **Authors' contributions***

IMH, JØH, MØ, and LTM contributed to the conceptualization and study design. IMH, JØH, LTM, GHG, and RMÅ contributed to sample collections. RMÅ conducted enzyme activity analyses, 16S rRNA extraction, and sequencing. IMH and GHG performed statistical analysis on growth performance, liver index, fecal consistency, and digestibility data. IMH performed statistical analysis on pH, enzyme activities, short-chain fatty acids, and raw sequence data. IMH wrote the original draft. All authors critically reviewed the original draft and approved the final manuscript.

*5.2. **Conflict of interests***

The authors declare that they have no competing interests.

5.3. Highlights:

- Inclusion of up to 19% full-fat BSFL meal did not affect performance results.
- Increased inclusion of BSFL decreased CTTAD of crude protein.
- Increased inclusion of BSFL increased CTTAD of crude fat.
- Dietary inclusion of BSFL reduced the relative abundance of *Lactobacillus* in colon.