



Yellow mealworms (*Tenebrio molitor*) as an alternative animal feed source: A comprehensive characterization of nutritional values and the larval gut microbiome

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ABSTRACT

The present study aimed to evaluate the potential of yellow mealworms (*Tenebrio molitor*) reared using local agricultural by-products as an alternative feed for monogastric and ruminant animals. The mealworms were raised on oat-based (OB) and wheat-based (WB) by-products, and their nutritional properties and *in vitro* digestibility were evaluated, simulating the digestive system of both monogastric and ruminant animals. Furthermore, the gut microbiome of mealworm larvae was studied. Crude fat and most minerals were higher in larvae fed WB than those fed OB ($P < 0.05$), reflecting the nutritional profiles of the substrates. Larvae and pupae generally shared a common nutritional profile: lower contents of crude fiber, crude protein, and total amino acids, and higher crude fat, total fatty acids, and gross energy levels compared to adults ($P < 0.05$). Total essential and non-essential amino acid contents in larvae and pupae were similar to those of a commercial soybean meal (SBM). The *in vitro* dry matter and protein digestibility of larvae and pupae were similar to SBM and significantly higher (30%) than the values for adults for both monogastrics and ruminants. Firmicutes and Proteobacteria were the most abundant gut microbial phyla in larvae, and the gut microbiome revealed remarkable plasticity in response to altered nutritional status, such as starvation. A new insight into the nutrition of mealworm's metamorphic stages fed on agricultural by-products and how feeding modulates the larval gut microbiome provides an innovative approach to exploit mealworms as a sustainable and alternative animal feed source in the future.

1. Introduction

The world's human population is growing rapidly, and ensuring food security is a global concern. The livestock sector plays a vital role in agricultural food production, contributing 15% of total food energy and 31% of dietary protein globally (Godde et al., 2021; Raney et al., 2009). Due to increased calorie intake and the nutritional shift toward animal-based products worldwide, it is anticipated that future demands for livestock-based products will increase even further, particularly in

low- and middle-income countries (Alexandratos and Bruinsma, 2012; Enahoro et al., 2018). Such increased demands for livestock products can be fulfilled through the identification and utilization of alternative animal feeding resources, as they play a crucial role in establishing a sustainable livestock sector in the future (Eisler et al., 2014; Pinotti et al., 2021). In recent years, different insect species have been identified as promising alternative and more sustainable feed ingredients for livestock due to their capability to convert waste or by-products into biomass rich in protein and other valuable nutrients (Adhikari et al.,

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2021; Veldkamp and Bosch, 2015).

Among insects, the yellow mealworm (*Tenebrio molitor*) has been identified as one of the few candidate species with the potential for large-scale commercial production (Heckmann et al., 2018). Mealworms can potentially be grown under a variety of agricultural and other low-quality organic substrates (Ruschioni et al., 2020; Van Broekhoven et al., 2015; Zhang et al., 2019) and can be utilized as alternative nutrient sources for livestock, mainly monogastric animals (Hong et al., 2020; Veldkamp and Bosch, 2015). In Nordic countries, self-sufficiency for domestic animal feed resources is relatively low (Åby et al., 2014). Thus, the local production and utilization of new feed ingredients, such as mealworms, would contribute to an increased domestic supply of raw ingredients for feed industries in the region. This requires knowledge of whether locally available bioresources, such as agricultural by-products from the Nordics, can be efficiently utilized to produce mealworm biomass of high nutritional quality for production animals.

The ability of mealworm larvae to use various substrates may depend upon the interaction between the larvae and their gut microbiome. For example, specific gut bacterial strains of the genera *Klebsiella*, *Pseudomonas*, and *Serratia* found in mealworm larvae were linked to bioplastic biodegradation (Urbanek et al., 2020). Furthermore, a substantial change in the larval intestinal microbial community of mealworms was observed with dietary modification, i.e., *Lactobacillus* and *Mucispirillum* were associated with a plastic-enriched bran diet (Lou et al., 2021). However, changes in the gut microbial communities of mealworm larvae in response to an altered short-term nutritional status are largely unknown. This study investigates the potential changes in the gut microbial population of mealworm larvae exposed to nutritional challenges. Such data would be important in determining whether the gut microbiome could be used to improve the feed efficiency of mealworm larvae in commercial mealworm farming.

The mealworm is a holometabolous species, and its life cycle consists of four distinct metamorphic stages: egg, larva, pupa, and adult. Previously, the biomass of mealworm larvae has been investigated in terms of their potential nutritional value as feed for animals. For example, including mealworm larvae in the diets of pigs and poultry improved their nutrient digestibility and growth performance (Jin et al., 2016; Biasato et al., 2017). It is noteworthy that mealworms, at their different metamorphic stages, can have unique chemical compositions; for example, the level of chitin can affect the potential of utilizing them as animal feed ingredients (Shin et al., 2019; Song et al., 2018). However, the impacts of such metamorphic stages on the nutritional profile and their utilization as animal feed ingredients have often been overlooked. This study aims to characterize the metamorphic-stage-specific nutritional compositions and *in vitro* digestibility (for both monogastric and ruminant animals) of mealworms.

The objectives of this study were to test the hypotheses that a) high nutritional quality of mealworms is achieved by using agricultural by-products that are locally available in the Nordic region, b) nutritional value and digestibility of mealworms are dependent on their metamorphic stages, and c) short-term nutritional challenges modulates the gut microbiome of mealworm larvae.

2. Methodology

2.1. Mealworm rearing and production

Mealworm production was conducted at the Mealworm Production Facility of the Faculty of Biosciences and Aquaculture of Nord University situated at Mære Agriculture School (Mære Landbruksskole, Sparbu. All research activities in mealworms were carried out following standard ethical procedures. The mealworm larvae were reared on two different feeding substrates: oat by-product (OB; oatmeal from oat grain not suitable for human consumption; ~1 mm particle size) and wheat by-product (WB; wheat bran; ~1 mm particle size; Hvetekli, Gullimunn AS, Steinkjer, Norway) (Tables 1 and 2). These by-products were chosen

Table 1

The chemical and nutritional compositions (dry matter, DM, basis) of different feeding substrates used to produce mealworms.

Parameters	OB	WB	P value
Ash (% DM)	2.1 ± 0.08 ^b	3.4 ± 0.07 ^a	<0.0001
CP (% DM)	12.0 ± 0.21 ^b	14.0 ± 0.18 ^a	<0.0001
NFE (% DM)	53.2 ± 1.22 ^b	61.4 ± 1.03 ^a	0.0004
CF (% DM)	12.5 ± 0.19 ^a	5.1 ± 0.16 ^b	<0.0001
EE (% DM)	3.0 ± 0.13	3.3 ± 0.11	0.11
GE (MJ/kg DM)	19.4 ± 0.03 ^a	18.8 ± 0.03 ^b	<0.0001

Results are presented as mean ± standard errors of the mean. Treatment groups with different letters in the superscripts within a row are significantly different ($P < 0.05$). OB, oat-based by-products; WB, wheat-based by-products; CP, crude protein; NFE, nitrogen-free extract; CF, crude fiber; EE, ether extract; GE, gross energy.

Table 2

The mineral profile (dry matter, DM; basis) of different feeding substrates used to produce mealworm.

Minerals	OB	WB	P value
Na (% DM)	0.002 ± 0.0013	0.004 ± 0.0008	0.3090
Mg (%DM)	0.13 ± 0.020 ^b	0.29 ± 0.013 ^a	0.0001
K (%DM)	0.50 ± 0.054 ^b	0.87 ± 0.035 ^a	0.0050
Ca (%DM)	0.077 ± 0.0027 ^a	0.062 ± 0.0019 ^b	0.0025
Mn (mg/kg DM)	58.7 ± 3.76	68.8 ± 2.66	0.0633
Fe (mg/kg DM)	70.0 ± 1.94 ^b	77.4 ± 1.27 ^a	0.0130
Cu (mg/kg DM)	7.7 ± 0.56	9.0 ± 0.37	0.0839
Zn (mg/kg DM)	29.8 ± 4.6 ^b	48.7 ± 3.01 ^a	0.0090

Results are presented as mean ± standard errors of the mean. Treatment groups with different letters in the superscripts within a row are significantly different ($P < 0.05$). OB, oat-based by-products; WB, wheat-based by-products.

based on their local availability. Mealworm larvae production was initiated by setting up breeding, providing feeding substrates (400 g) ~500 mealworm adults (60–65 g) for two weeks in a plastic tray (53.5 × 34.5 × 5.6 cm; Witre AS, Halden, Norway; Fig. 1) maintained under a production rack (60 × 40 × 180 cm; Runex Mjøndalen, Norway; Fig. 1). Afterward, the adults (beetles) were removed from the tray, additional fresh substrates (600 g) were provided in the tray, and the larvae were allowed to grow until ~10 weeks of age before they were harvested, as mentioned below.

For assessing the nutritional composition and digestibility of different metamorphic stages of mealworms (larvae, pupae, and adults), only larvae fed with WB were allowed to develop into pupae and beetles because WB was readily available and did not require any pre-processing before using it as a rearing substrate for mealworms. A commercial soybean meal (SBM; LabTek, Ås, Norway) was also included to compare the nutritional values of mealworms in different metamorphic stages. All mealworm experiments, including breeding, were performed at least in triplicate. Carrots were used as a source of water for both larvae and adults.

2.2. Mealworm harvesting and processing

Feed was removed 24 h before harvesting the mealworms, except for sampling larvae for gut microbiome analysis in a fed condition. For chemical and nutritional analyses, ~10 weeks old larvae were harvested by sieving. Pupae and adults were collected from the WB-fed group. The harvested larvae, pupae, and adults were cleaned with regular tap water, frozen at −20 °C, and freeze-dried (VWR, FREEZONE 8L, Labconco Corporation, Kansas City, MO, USA) for at least 72 h (−50 °C, <0.133 mbar). The samples were then ground, and various analyses were performed.

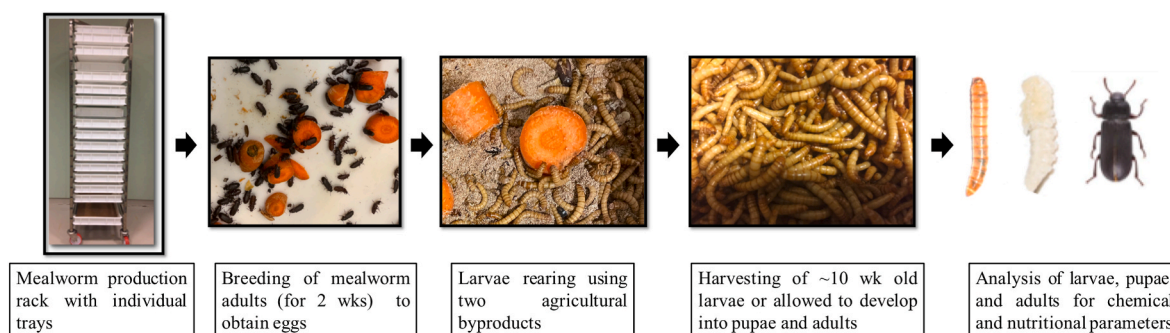


Fig. 1. Schematic diagram of experimental outline. Mealworms were grown in trays feeding two different agricultural by-products (OB, oat-based; WB, wheat-based) under a production rack. After ~10 weeks, larvae were harvested, processed, and analyzed for various chemical and nutritional parameters. In addition, the nutritional profiles of pupae and adults were characterized and compared with a commercial soybean meal.

2.3. Chemical analyses of feeds and mealworms

Feed and insect samples were analyzed for their chemical compositions according to official methods. Dry matter (DM) was determined by drying samples at 105 °C for 24 h (ISO 6496: 1999), and ash content was determined by weighing the residue after combustion at 550 °C overnight (ISO 5984: 2002). Crude protein (CP) was calculated from analyzed Kjeldahl N (AOAC, 2001; Kjeltex™ 8400, FOSS Denmark, Hillerød, Denmark), where different conversion factors were used for substrates (5.83), mealworms (4.76) and SBM (5.71) as previously suggested (McDonald et al., 2011; Janssen et al., 2017). Crude fat (ether extract; EE) was analyzed after extraction with 80% petroleum ether and 20% acetone in an Accelerated Solvent Extractor from Dionex (ASE200; Sunnyvale, CA, USA) (Commission Regulation (EC) No 152/2009). Gross energy (GE) content was determined using a Bomb Calorimeter (PARR 6400, PARR Instruments, Moline, IL, USA) (ISO 9831, 1998). The crude fiber content (CF) was determined by sequentially treating the samples with H₂SO₄ (1.25%) and NaOH (1.25%) (Ankom200 Fiber Analyzer, NY, USA), and the organic matter (OM) of the residue obtained after combustion.

2.4. Mineral profile of feed and mealworms

The mineral content was determined after a pre-digestion of 150 mg of feed or insect samples with a mixture of concentrated HNO₃ and hydrogen peroxide (5:1, v/v) using a microwave digestion system (Milestone Srl, Sorisole BG, Italy). Macrominerals (Sodium, Na; Potassium, K; Calcium, Ca; Magnesium, Mg) and trace minerals (Manganese, Mn; Iron, Fe; Zinc, Zn; Copper, Cu) were determined spectrophotometrically using a microwave plasma atomic emission spectrometer (MP-AES 4200, Agilent Technologies) following standard protocols (Commission, 2009). Concentrations of minerals were determined using a calibration curve.

2.5. Amino acid profile of mealworms

Following the EU commission regulation (EC, No. 152/2009), the total amino acid (except tryptophan) content (peptide-bound and free) of the mealworms was analyzed using a Biochrom 30 Amino Acid Analyzer (Biochrom Ltd., Cambridge, UK) with ion exchange column, post-column derivatization, and photometric detector (Commission, 2009): cysteine (Cys), methionine (Met), aspartic acid (Asp), threonine (Thr), serine (Ser), glutamic acid (Glu), proline (Pro), glycine (Gly), alanine (Ala), valine (Val), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), histidine (His), lysine (Lys), and arginine (Arg). This method leads to the deamination of two amino acids: asparagine and glutamine, turning them into aspartic and glutamic acids, respectively (Smith, 2017). Thus, the levels of aspartic acid (Asp) and glutamic acid (Glu) presented in this study also account for the values of

asparagine and glutamine, respectively. The total tryptophan content was determined by basic hydrolysis and separation by high-performance liquid chromatography (HPLC) and fluorometric detection (UltiMate 3000 – Detector, Dionex Softron GmbH, Germering, Germany) (Commission, 2009).

2.6. Fatty acid profile of mealworms

The fatty acid contents of mealworms were determined by first releasing fatty acids from the glycerol unit by saponification and then derivatizing them into fatty acid methyl esters (FAME) before they were extracted into heptane as previously described (O'Fallon et al., 2007). The FAMES were then quantified by gas chromatography using an auto-injector (TRACE™ GC Ultra, Thermo Scientific, Massachusetts, USA), with the following specifications: column: Rt-2560, 100 m, 0.25 mm ID, 0.20 µm dt; injector temperature: 250 °C; split injection: 1:40 split ratio; injection volume: 1 µL; carrier gas: helium; constant pressure: 2.70 bar; oven temperature: 140 °C (5 min) to 240 °C at 4 °C/min; detector: FID, temperature 250 °C; analysis time: 50 min.

2.7. Carbohydrate profile of mealworms

Mealworm samples were hydrolyzed, and subsequent monomeric moieties were analyzed using high-performance anion exchange (HPAE) chromatography, as previously reported (Janssen et al., 2017). In summary, an ICS-5000 ion chromatography HPLC system (a Dionex CarboPac PA-1 column; 2 × 250 mm in combination with a Dionex CarboPac PA guard column; 2 × 25 mm) with a pulsed electrochemical detector (in pulsed amperometric detection mode) was used (ThermoFisher Scientific, Breda, The Netherlands). Monosaccharides were detected after the post-column addition of 0.5 M sodium hydroxide (0.10 mL min⁻¹). Elution was carried out at 15 °C.

2.8. Determination of the total polyphenol content (TPC) of mealworms

2.8.1. Extraction of total polyphenols

Total polyphenols in the dried mealworm powder were extracted using a two-step procedure as previously described (Pandey et al., 2022) with some modifications. In brief, 20 mL of 50% methanol-water (1:1 v/v) solution (Merck KGaA, Darmstadt, Germany) was added to 1 g of mealworm (20:1 v/w) and shaken at 150 rpm for 2 h at room temperature in the dark. The mixture was centrifuged at 12000×g for 10 min, and the supernatant was recovered in a separate test tube. The residue was reextracted in 20 mL of 70% acetone-water solution (7:3 v/v) (Merck KGaA, Darmstadt, Germany). The supernatants of both extractions were pooled and filtered (Whatman™ grade 597 standard filter paper, Cytiva Europe GmbH, Freiburg, Germany). The filtered solution was used as a crude polyphenol extract to quantify TPC. The extractions were performed in duplicates.

2.8.2. Quantification of the total polyphenol contents (TPC)

The TPC in the extracts and blanks was determined following the protocol previously described (Pandey et al., 2022), where 20 μL of crude polyphenol extracts, blanks, and standards were loaded in triplicates in a 96-well microplate (Thermo Fischer GmbH, Kandel, Germany). Then 100 μL Folin-Ciocalteu reagent was added to each well and mixed properly by gentle shaking. After 5 min of incubation at room temperature, 80 μL of 7.5% sodium carbonate solution (Thermo Fischer GmbH, Kandel, Germany) was added, mixed by gentle shaking, and the plate was incubated in the dark for 2 h at room temperature. To generate a standard curve, seven different concentrations of gallic acid (500, 250, 125, 62.5, 31.25, 15.625, and 0 $\mu\text{g mL}^{-1}$; Merck KGaA, Darmstadt, Germany) were included in the assay. TPC concentrations were determined based on absorbance at $\lambda 750\text{ nm}$ using a spectrophotometric microplate reader (BIO-RAD, iMark™ Microplate Reader, California, USA).

Mean TPCs were expressed as milligrams of gallic acid equivalents (mg GAE) per gm DM:

$$\text{TPC (mg GAE)} = \frac{(\text{Mean TPC of sample } \mu\text{g} / \text{mL} \times \text{Solvent volume})}{\text{DM weight of sample (g)} \times 1000} \times 100\%$$

2.9. In vitro digestibility of mealworms

2.9.1. In vitro digestibility for ruminant animals

In vitro digestibility in ruminant animals was determined following the method of Tilley and Terry (1963), modified by Goering and Van Soest (1970), using ruminal fluid from two nonlactating Holstein cows fitted with rumen cannula (10 cm diameter; Bar Diamond Inc., Parma, ID), and housed on the Vairão Agricultural Campus of the School of Medicine and Biomedical Sciences of the University of Porto (ICBAS-UP, Vila do Conde, Portugal). The care and management of the cows followed the good animal practices of the European Union (Directive, 2010/63/EU). All animal procedures and methodologies were reviewed and approved by the Animal Ethics Committee of ICBAS-UP, licensed by the Portuguese Directorate General of Food and Veterinary Medicine (permit #FT2014DGV 046412 ICB), and performed by trained scientists (FELASA category C). The cows were fed a total mixed ration comprising, on a DM basis, 56% corn silage, 11% haylage, 16% wheat straw, and 17% compound feed (524 g kg^{-1} DM; 248 g kg^{-1} starch, 460 g kg^{-1} NDF, and 91 g kg^{-1} CP, DM basis) at 08:00 and 18:00 h and had free access to fresh drinking water. Ruminal fluid was collected before feeding in the morning, strained through four layers of cheesecloth, and kept at 39 °C under CO_2 . Two hundred and fifty mg of each sample, a laboratory reference sample (soybean meal, 1 mm ground), and blanks were incubated in quadruplicate. Each sample was incubated in 50 mL centrifuge tubes with 25 mL buffered rumen fluid solution (1 strained rumen fluid:4 Kansas State buffer) (Marten and Barnes, 1979) flushed with O_2 -free CO_2 , and closed with rubber stoppers fitted to a Bunsen valve to control the pressure build-up of gases in the headspace from fermentation. The tubes were incubated for 48 h at 39 °C in a water bath. Afterward, the incubation contents were filtered through a fritted crucible (porosity 40–100 μm , P2), and the residues were extracted in boiling neutral detergent solution (Robertson, 1981) for 1 h. Crucibles were dried at 103 °C overnight and weighed to calculate *in vitro* DM digestibility as the difference between the incubated DM and the non-digested DM, considered as the residue that remained in the crucibles. The samples were corrected for bacterial and residual DM by subtracting the blanks. Two crucibles from each sample were incinerated in a muffle furnace at 500 °C for 3 h for the calculation of OM digestibility, and the other two crucibles were used to determine the N content in the residues using a Leco nitrogen analyzer (Model FP-528, Leco Corporation, St. Joseph, USA) for the calculation of CP digestibility. Rumen fluid blanks were used to correct the OM and CP digestibility of samples.

2.9.2. In vitro digestibility for monogastric animals

A three-step enzymatic *in vitro* method was used to determine the *in vitro* digestibility of DM, OM, and CP of experimental samples for monogastric animals (Boisen and Fernández, 1997). Each mealworm sample, a laboratory reference sample (soybean meal, 1 mm ground), and blanks were incubated in quadruplicate. Briefly, samples (500 mg) were placed in 100 mL flasks, and 25 mL of phosphate buffer (0.1 M, pH 6.0) and 10 mL of 0.2 M HCl were added. The pH of the mixture was adjusted to 2.0, and 1 mL of a freshly prepared pepsin solution containing 25 mg pepsin per mL (pepsin from porcine gastric mucosa, 2000 U/g, Merck 1.07190.1000) was added. To prevent bacterial growth, 0.5 mL of chloramphenicol solution (0.5 g in 100 mL of ethanol) was added. The capped flasks were incubated in a water bath at 39 °C for 2 h with agitation. Once at room temperature, 10 mL of phosphate buffer (0.2 M, pH 6.8) and 5 mL of 0.6 M NaOH solution were added to the flask, and the pH was adjusted to 6.8. One mL of a freshly prepared pancreatin solution containing 100 mg of pancreatin per mL (porcine, grade IV, reference Sigma Aldrich P-1750) was added to the mixture, and the flasks were incubated in a water bath at 39 °C for 4 h with agitation. The flasks were allowed to cool at room temperature, and 10 mL of 0.2 M EDTA solution was added. The pH of the mixture was adjusted to 4.8 with an acetic acid solution (30%, v/v) and 0.5 mL of a mixed multi-enzymatic complex containing arabinase, cellulase, β -glucanase, hemi-cellulase, xylanase, and pectinase (Viscozyme L, Sigma-Aldrich V2010) was added. Flasks were incubated in a water bath at 39 °C for 18 h under agitation. The undigested residue was transferred to the crucibles (porosity 40–100 μm , P2) by filtration and rinsed with ethanol and acetone. The crucibles were dried at 103 °C overnight and weighed for the *in vitro* DM digestibility calculation. For the OM digestibility calculation, two crucibles of each sample were incinerated in a muffle furnace at 500 °C for 3 h. The CP (N \times 6.25) was determined using a Leco nitrogen analyzer (Model FP-528, Leco Corporation, St. Joseph, USA) with the remaining two residues from each sample. *In vitro* digestibility was calculated as the difference between the DM, OM, or CP in the sample and the undigested residue after correction for the blank.

2.10. Gut microbiome analyses of mealworm larvae using 16S rRNA gene amplicon sequencing

At the end of the feeding period, 8–10 larvae from the WB-fed group were randomly selected in both fasted (for 24 h) and fed (remained fed while sampling) conditions. The fed or fasted larvae were used for their gut collection as previously described (Lou et al., 2021) with some modifications. First, all larvae were immobilized by placing them on ice, were sterilized with 70% ethanol for ~1 min, rinsed twice with sterile Type I water, and the entire gut was sampled and stored at –80 °C until further processing.

2.10.1. DNA extraction

The frozen samples were thawed in ice, and the individual gut samples were transferred to a new sterile tube. The DNA from the gut samples was extracted using the FastDNATM SPIN Kit for Soil (MP Biomedicals, California, USA) and further purified using the Monarch® PCR & DNA Cleanup Kit (New England Biolabs Inc., Ipswich, MA, USA) following the manufacturer's protocols. The concentration and purity of the extracted DNA were tested with a NanoDrop Lite UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.10.2. Library preparation

The V4 regions of the bacterial 16S-rRNA gene were amplified using universal primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2011). For amplicon library preparation, 30 ng of microbial DNA templates and 16S rRNA fusion primers were mixed and subjected to a polymerase chain reaction (PCR). The conditions of the PCR were 95 °C for 3 min, 30 cycles: 95 °C for 45 s, 56 °C for 45 s, 72 °C for 45 s, a final elongation at 72 °C for 10

min, and 4 °C hold. All PCR products were purified by Agencourt AMPure XP beads and dissolved in the elution buffer. Subsequently, a one-step PCR was performed to add 12 nt-Golay barcodes and Illumina P5 and P7 adapter sequences to complete the library construction (Rapin et al., 2017). The size and concentrations of the libraries were determined using the Agilent 2100 Bioanalyzer, and the qualified libraries were sequenced on the Illumina HiSeq 2500 platform (Illumina Inc., California, USA) using the paired-end 250-bp dual index at the facility of BGI Tech Solutions (Hongkong). The sequenced files have been deposited in the NCBI Sequence Read Archive (SRA) and can be accessed under the accession number: PRJNA890055.

2.10.3. Bioinformatics and data analysis of sequencing data

All bioinformatics analyses of sequencing data were performed as previously reported (Pandey et al., 2022). Briefly, DNA reads obtained from the HiSeq run were analyzed using QIIME2 (Bolyen et al., 2019) and the dada2 plugin (Callahan et al., 2016). The paired-end reads were denoised, joined, dereplicated, the forward and reverse primers trimmed, and finally filtered for the chimeras using the 'dada2 denoise-paired' command. Subsequently, the taxonomy for amplicon sequence variants (ASV) was assigned through 'feature-classifier classify-consensus-vsearch' using the SILVA 138 database (Quast et al., 2012). The ASV table and taxonomy files were imported into R version 4.0.3 (Team, 2021) to perform data visualization and further analysis. Diversity-based analysis was performed using the 'vegan' package ver. 2.5–7 (Oksanen et al., 2021) and 'phyloseq' package ver. 1.34 (McMurdie and Holmes, 2013). The ASV table was transformed to relative abundance for beta diversity, and the dissimilarity matrices were visualized using principal coordinates analysis (PCoA). For the PERMANOVA test and partitioning of variance, the adonis test was carried out. Differential ASV abundance analysis using the 'DeSeq2' package was used to identify significantly different microbial taxa between the fed and non-fed larval gut microbiome.

2.11. Calculations

2.11.1. Nitrogen-free extract (NFE)

Nitrogen-free extract, an indicator of soluble carbohydrates, of feed or mealworm was calculated as follows:

$$\text{NFE (\%)} = \text{DM (\%)} - \text{CF (\%)} - \text{CP (\%)} - \text{EE (\%)} - \text{Ash (\%)}$$

Where NFE, nitrogen-free extract; DM, dry matter; CF, crude fiber; CP, crude protein; EE, ether extract.

2.11.2. Essential amino acid index (EAAI)

The essential amino acid index (EAAI) of mealworms and SBM was calculated as follows (Veldkamp and Bosch, 2015; Smith, 2017):

$$\text{EAAI} = \sqrt[n]{\frac{aa_1}{AA_1} \times \frac{aa_2}{AA_2} \times \dots \times \frac{aa_n}{AA_n}}$$

Where, aa, the amount of specific essential amino acid expressed as a percentage of CP; AA, the requirement for the same amino acid for the target animal (growing piglets) expressed as a percentage of CP; n, the total number of amino acids used in the calculation.

2.11.3. Chemical score

The chemical score (CS) of mealworms and SBM was calculated as follows (Rao et al., 1959; Veldkamp and Bosch, 2015):

$$\text{CS} = \frac{\text{EAA}}{\text{AAR}} \times 100$$

Where EAA, an essential amino acid expressed as a percentage of CP; AAR, the animal requirement of the EAA for a target animal (growing piglets) expressed as a percentage of CP. The essential amino acid with the lowest CS value was considered the first limiting essential amino

acid.

2.12. Statistical analyses

All statistical analyses were performed in R (The R Foundation for Statistical Computing Platform, version 4.0.2) using a general linear model (lm function). The model included substrate or mealworm metamorphic stage as fixed effects and the residual error. The homogeneity of the variance was evaluated by visual inspection of residual plots and their patterns, and the normality of the residuals was tested by quantile-quantile plots. All values were averaged across the mealworm stages or SBM while generating a heatmap for the hierarchical clustering of amino acid and fatty acid profiling data. The values were center-scaled (z-transformation), and in the end, the Euclidean distance matrix was generated, and the "centroid" algorithm was conducted for hierarchical agglomerative clustering using packages ComplexHeatmap (Gu et al., 2016) and dendextend (Galili, 2015) in R. Differences in least-square means (LS means) were compared using Tukey's multiple comparison test, and the results are expressed as LS means with the standard error of the mean (LS means \pm SEM). The level of significance was set at $P < 0.05$.

3. Results

3.1. Nutritional characteristics of rearing substrates

Ash ($P < 0.0001$), CP ($P < 0.0001$), and NFE ($P = 0.0004$) contents were higher in WB compared to the OB substrate (Table 1). Additionally, EE contents were numerically higher in WB than in OB. Conversely, CF (2-fold; $P < 0.0001$) and GE ($P < 0.0001$) contents of OB were higher than in WB.

The contents of both macro-and micro-minerals, except for Ca, were higher in WB than in OB (Table 2). In particular, the levels of Mg ($P = 0.0001$), K ($P = 0.005$), Fe ($P = 0.01$), and Zn ($P = 0.009$) were significantly higher in WB than the levels in OB. In contrast, the Ca content was significantly higher in OB compared to the level in WB ($P = 0.0025$).

3.2. Nutritional characteristics of larvae fed two different substrates

Only two nutritional parameters that were different in the larvae fed two different substrates were the EE ($P = 0.0259$) and ash ($P = 0.065$) contents, and they were higher in the larvae fed WB compared to those reared in OB (Table 3).

The effect of the rearing substrate on the larval Mg, Ca, and Zn contents followed the mineral composition of the substrates (Table 4). The Mg ($P < 0.001$) and Zn ($P = 0.0119$) levels were higher in the larvae fed WB compared to those provided OB. On the contrary, the Ca content was higher in the larvae fed OB compared to the larvae fed WB ($P = 0.007$).

Table 3

The chemical and nutritional compositions (dry matter, DM, basis) of mealworm larvae produced using different feeding substrates.

Parameters	OB-fed	WB-fed	P value
Ash (% DM)	4.91 \pm 0.176	5.42 \pm 0.176	0.0650
CP (% DM)	41.7 \pm 0.94	40.2 \pm 0.94	0.2875
NFE (% DM)	15.3 \pm 1.45	13.7 \pm 1.45	0.4616
CF (% DM)	7.78 \pm 0.321	7.57 \pm 0.321	0.6458
EE (% DM)	27.2 \pm 1.21 ^b	31.6 \pm 1.10 ^a	0.0259
GE (MJ/kg DM)	27.2 \pm 0.316	27.3 \pm 0.316	0.8360

Results are presented as mean \pm standard errors of the mean. Treatment groups with different letters in the superscripts within a row are significantly different ($P < 0.05$). OB, oat-based by-products; WB, wheat-based by-products; CP, crude protein; NFE, nitrogen-free extract; CF, crude fiber; EE, ether extract; GE, gross energy.

Table 4

The mineral profiles (dry matter, DM, basis) of mealworm larvae produced using different feeding substrates.

Minerals	OB-fed	WB-fed	P value
Na (% DM)	0.15 ± 0.009	0.15 ± 0.009	1.0000
Mg (% DM)	0.41 ± 0.0137 ^b	0.55 ± 0.0137 ^a	<0.001
K (% DM)	1.14 ± 0.038	1.15 ± 0.038	0.8085
Ca (% DM)	0.077 ± 0.004 ^a	0.057 ± 0.004 ^b	0.0073
Mn (mg/kg DM)	14.5 ± 0.439	14.0 ± 0.439	0.3880
Fe (mg/kg DM)	46.6 ± 3.52	49.3 ± 3.52	0.6001
Cu (mg/kg DM)	21.4 ± 0.788	20.2 ± 0.788	0.2897
Zn (mg/kg DM)	88.6 ± 5.13 ^b	110.8 ± 5.13 ^a	0.0119

Results are presented as mean ± standard errors of the mean. Treatment groups with different letters in the superscripts within a row are significantly different ($P < 0.05$). OB, oat-based by-products; WB, wheat-based by-products.

3.3. Nutritional characteristics of mealworms at different metamorphic stages

Ash contents of larvae, pupae, and adults were lower than those in SBM ($P < 0.0001$ for all; Table 5). Within mealworms, the ash content was higher in larvae than in pupae ($P = 0.039$) and adults ($P = 0.006$), whereas pupae and adults had similar values. Both CP and CF contents were higher in adults than in larvae ($P < 0.0001$ for both), pupae ($P < 0.0001$ for both), and SBM ($P < 0.0001$ for CF only). The CP contents in larvae ($P = 0.001$) and pupae ($P = 0.003$) were lower than the values in SBM, but larvae and pupae had similar CP contents. The contents of CF in larvae and SBM were identical, but pupae had significantly lower CF than larvae ($P = 0.001$). The EE and GE contents in larvae and pupae were higher than in adults ($P < 0.0001$ for all) and SBM ($P < 0.0001$ for all), but the values were similar in the larvae and pupae. In addition, EE and GE levels were significantly higher in adults than in SBM ($P < 0.0001$ for both).

3.4. Carbohydrate profile of mealworms at different metamorphic stages

The amount of fucose, galactose, xylose, and mannose varied significantly in larvae, pupae, and adults (Table 6). Fucose, arabinose, and xylose were significantly higher in larvae than in pupae and adults ($P < 0.05$ for all). Glucosamine was lower in pupae than in adults and larvae ($P < 0.05$ for all). Mannose content was significantly different ($P = 0.0037$) among larvae, pupae, and adults, with the highest values in pupae, followed by adults and larvae. Glucose and galactose did not differ significantly with the metamorphic stages.

Table 5

The chemical and nutritional compositions (dry matter, DM) of different metamorphic stages of the mealworm raised under a wheat-based (WB) substrate.

Parameters	Larvae	Pupae	Adults	SBM	P value
Ash (% DM)	4.58 ± 0.24 ^b	3.59 ± 0.24 ^c	3.3 ± 0.24 ^c	10.4 ± 0.45 ^a	<0.0001
CP (% DM)	40.0 ± 0.50 ^b	40.4 ± 0.54 ^b	47.2 ± 0.50 ^a	44.9 ± 0.93 ^a	<0.0001
NFE (% DM)	15.8 ± 1.37 ^b	16.7 ± 1.48 ^b	7.68 ± 1.37 ^c	30.55 ± 2.57 ^a	<0.0001
CF (% DM)	6.81 ± 0.71 ^b	3.11 ± 0.71 ^c	20.64 ± 0.71 ^a	5.36 ± 1.33 ^b	<0.0001
EE (% DM)	29.5 ± 0.96 ^a	28.5 ± 0.956 ^a	18.47 ± 0.956 ^b	1.36 ± 1.788 ^c	<0.0001
GE (MJ/kg DM)	27.5 ± 0.185 ^a	28.0 ± 0.185 ^a	25.6 ± 0.185 ^b	18.7 ± 0.346 ^c	<0.0001

Results are presented as mean ± standard errors of the mean. Treatment groups with different letters in the superscripts within a row are significantly different ($P < 0.05$). CP, crude protein; NFE, nitrogen-free extract; CF, crude fiber; EE, ether extract; GE, gross energy; SBM, soybean meal.

Table 6

Carbohydrate profile (% dry matter, DM) of mealworms at different metamorphic stages raised under a wheat-based substrate.

Carbohydrates	Larvae	Pupae	Adults	P value
Fucose	0.105 ± 0.003 ^a	0.06 ± 0.003 ^b	0.06 ± 0.003 ^b	0.002
Arabinose	0.185 ± 0.015 ^a	0.035 ± 0.015 ^b	0.085 ± 0.015 ^b	0.0128
Galactose	0.085 ± 0.015	0.025 ± 0.015	0.065 ± 0.015	0.1386
Glucose	2.56 ± 0.693	3.6 ± 0.693	2.67 ± 0.693	0.5716
Glucosamine	3.96 ± 0.217 ^a	2.1 ± 0.217 ^b	4.68 ± 0.217 ^a	0.0075
Xylose	0.18 ± 0.006 ^a	0 ± 0.006 ^b	0.005 ± 0.006 ^b	0.0004
Mannose	0.115 ± 0.0212 ^c	0.445 ± 0.0212 ^a	0.31 ± 0.0212 ^b	0.0037
Total Sugar	7.15 ± 0.72	6.26 ± 0.72	7.88 ± 0.72	0.395

Results are presented as mean ± standard errors of the mean. Treatment groups with different letters in the superscripts within a row are significantly different ($P < 0.05$).

3.5. Amino acid profile of mealworm at different metamorphic stages

Overall, the amino acid profile (also relative amino acid profile; Suppl. Figure 1) was clustered into three major groups, with larvae and pupae sharing a common cluster and adults and SBM forming separate groups (Table 7 and Fig. 2). Adults had a larger amount of both total and essential amino acids compared to those of larvae ($P < 0.0001$ for both), pupae ($P < 0.001$ for both), and SBM ($P = 0.059$, $P = 0.014$, respectively) (Table 7). Adults had higher levels of all essential amino acids (Phe, Val, Thr, Trp, Ile, Met, His, Leu, Lys) except Phe, Met, and Lys compared to those of larvae, pupae, and SBM. Phe was the only essential amino acid with significantly higher values in SBM than in mealworms ($P < 0.001$). His was significantly lower in SBM than in mealworms ($P < 0.01$ for all stages). The amounts of non-essential amino acids were also higher in adults than in larvae and pupae, except for the Cys, Asp, Glu, and Ser levels, which were the highest in SBM. Cys, Pro, and Ala levels were higher in larvae, but the rest of the non-essential amino acids shared similar values in larvae and pupae. The only amino acid that was significantly higher in pupae compared to larvae was Glu ($P = 0.0001$). Relative amounts of total essential or non-essential amino acids were similar in mealworms (all stages) and SBM (Suppl. Table 1).

3.6. Essential amino acid index (EAAI)

The metamorphic stage affected the EAAI of mealworms for growing piglets ($P < 0.0001$; Fig. 3). Pupae and larvae had similar EAAIs, but their levels were higher than those of adults ($P = 0.001$; $P = 0.002$, respectively) and SBM ($P = 0.001$; $P = 0.002$, respectively). The EAAIs in adults and SBM were found to be similar.

3.7. Chemical score

The chemical score of amino acids in mealworm larvae, pupae, adults, and SBM were calculated for growing piglets (Table 8). The first limiting amino acids identified in mealworm and SBM were Met and the combination of Met and Cys (Met + Cys) as the CS values were below 100. Adults had significantly lower CS values for Met than larvae ($P = 0.04$). In contrast, the values in larvae and pupae and SBM were not significantly different, although the values were numerically higher in larvae and pupae compared to SBM (~99 vs. ~88). The CS values for Met + Cys were significantly lower in adults than the values in larvae ($P = 0.0001$), pupae ($P = 0.011$), and SBM ($P = 0.006$), whereas the values in larvae, pupae, and SBM were similar.

3.8. Fatty acids profile of mealworm at different metamorphic stages

Mealworm larvae and pupae had similar fatty acid contents (Fig. 4; relative fatty acid profile: Suppl. Figure 2) but had higher levels of both

Table 7

The amino acid profile (g/kg dry matter, DM) of different metamorphic stages of the yellow mealworm raised under a wheat-based substrate.

Amino acids	Larvae	Pupae	Adults	SBM	P value
Phe	16.2 ± 0.423 ^b	17.0 ± 0.519 ^b	15.7 ± 0.480 ^b	22.3 ± 0.898 ^a	<0.0001
Val	20.0 ± 0.765 ^b	20.3 ± 0.937 ^b	25.1 ± 0.867 ^a	16.1 ± 1.622 ^b	0.00015
Thr	17.2 ± 0.228 ^b	17.6 ± 0.263 ^{ab}	18.1 ± 0.243 ^a	16.9 ± 0.455 ^{ab}	0.03052
Trp	4.59 ± 0.105 ^c	5.83 ± 0.141 ^b	6.43 ± 0.119 ^a	6.22 ± 0.223 ^{ab}	<0.0001
Ile	18.7 ± 0.268 ^b	19.0 ± 0.328 ^b	22.6 ± 0.304 ^a	19.3 ± 0.569 ^b	<0.0001
Met	5.38 ± 0.183	5.45 ± 0.224	5.30 ± 0.208	5.44 ± 0.389	0.9692
His	15.8 ± 0.271 ^b	16.1 ± 0.332 ^b	21.0 ± 0.307 ^a	12.2 ± 0.575 ^c	<0.0001
Leu	32.1 ± 0.484 ^b	33.3 ± 0.592 ^b	41.6 ± 0.548 ^a	33.7 ± 1.026 ^b	<0.0001
Lys	26.7 ± 0.791	26.2 ± 0.968	24.2 ± 0.896	26.7 ± 1.677	0.2222
Arg	27.0 ± 1.86	27.0 ± 2.28	27.5 ± 3.94	32.5 ± 7.70 ^b	0.6383
Tyr	30.2 ± 3.63 ^{ab}	27.8 ± 4.44 ^a	40.6 ± 4.11 ^a	14.1 ± 7.70 ^b	0.03107
Cys	3.93 ± 0.108 ^b	3.50 ± 0.133 ^c	3.74 ± 0.123 ^b	5.78 ± 0.23 ^a	<0.0001
Asp	34.2 ± 0.737 ^b	36.8 ± 0.902 ^b	34.5 ± 0.836 ^b	49.2 ± 1.563 ^a	<0.0001
Ser	18.9 ± 0.366 ^a	18.9 ± 0.366 ^a	17.2 ± 0.339 ^b	20.7 ± 0.634 ^a	<0.0001
Glu	53.9 ± 1.09 ^c	63.8 ± 1.33 ^b	52.1 ± 1.23 ^c	87.7 ± 2.3 ^a	<0.0001
Pro	29.9 ± 0.88 ^a	24.8 ± 1.078 ^b	32.2 ± 0.998 ^a	22.1 ± 1.867 ^b	<0.0001
Gly	20.8 ± 0.766 ^b	20.2 ± 0.939 ^b	38.2 ± 0.869 ^a	16.0 ± 1.626 ^b	<0.0001
Ala	30.5 ± 0.872 ^a	25.9 ± 1.068 ^b	33.4 ± 0.989 ^a	17.0 ± 1.850 ^c	<0.0001
Total AA	404 ± 5.52 ^b	409 ± 6.76 ^b	460 ± 6.26 ^a	424 ± 11.71 ^{ab}	<0.0001
EAA	155 ± 2.62 ^b	160 ± 3.21 ^b	180 ± 2.97 ^a	159 ± 5.55 ^b	<0.0001
NAA	245 ± 3.35 ^b	245 ± 3.35 ^b	276 ± 3.80 ^a	259 ± 7.11 ^a	<0.0001

Results are presented as mean ± standard errors of the mean. Treatment groups with different letters in the superscripts within a row are significantly different ($P < 0.05$). SBM, soybean meal; Phe, phenylalanine; Val, valine; Thr, threonine; Trp, tryptophan; Ile, isoleucine; Met, methionine; His, histidine; Leu, leucine; Lys, lysine; Arg, arginine; Tyr, tyrosine; Cys, cysteine; Asp, aspartic acid + asparagine; Ser, serine; Glu, glutamic acid + glutamate; Pro, proline; Gly, glycine; Ala, alanine; AA, amino acids; EAA, essential amino acids; NAA, non-essential amino acids.

saturated (SFA) and unsaturated fatty acids (UFA) than adults ($P < 0.001$ for all) and SBM ($P < 0.0001$ for all), whereas adults had higher values of both SFA ($P < 0.0001$) and USFA ($P < 0.0001$) than those of SBM (Table 9). In particular, the larvae and pupae had higher contents of palmitic (16:0), oleic (18:1n-9), and linoleic acid (18:2n-6) than in both adults and SBM. Furthermore, adults had higher palmitic, oleic, and linoleic contents than SBM. The main SFA in mealworms and SBM was palmitic acid (15–18%), while the most abundant UFAs in mealworms were oleic acid (44–69%) and linoleic acid (~25%). The most dominant UFAs in SBM were also linoleic (~54%) and oleic acid (~17%) (Suppl. Table 2). Regardless of their metamorphic stages, the mealworms contained similar relative amounts of fatty acid contents: ~75% UFA and ~25% SFA (Suppl. Table 2).

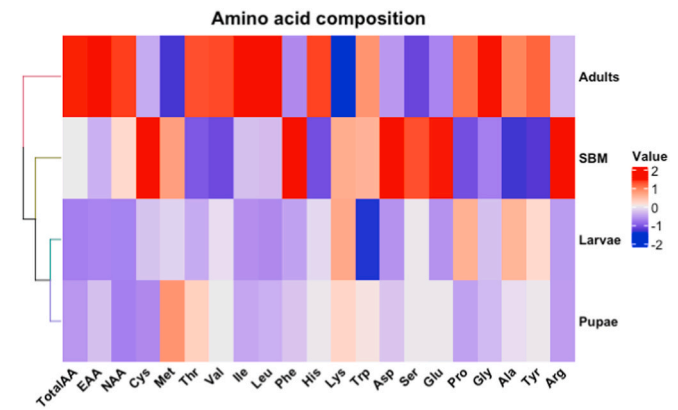


Fig. 2. Heatmap showing amino acid content among different metamorphic stages of mealworm compared to those in a soybean meal (SBM). Amino acid compositions are clustered into three distinct categories: 1) Adults, 2) SBM, and 3) Larvae and Pupae.

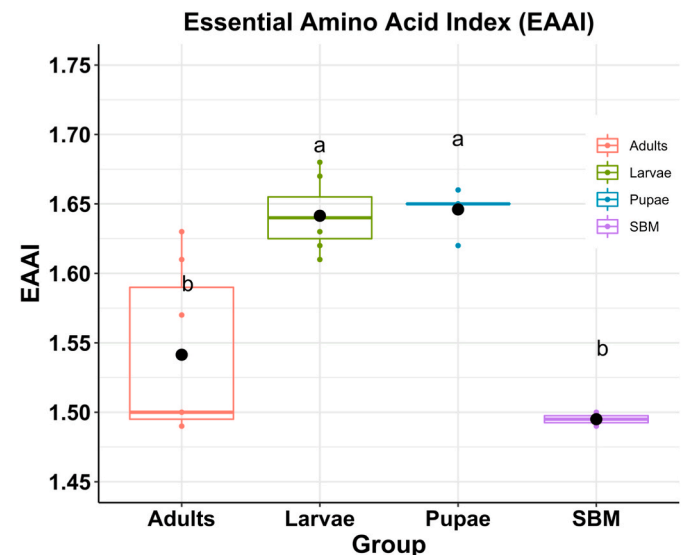


Fig. 3. Box plot representing Essential Amino Acid Index (EAAI) of different metamorphic stages of mealworm and soybean meal (SBM). EAAI was calculated as the amount of an amino acid expressed as a percentage of crude protein in mealworms, and SBM relative to the requirement for the same amino acid for the target animal (growing piglets) expressed as a percentage of crude protein of the target animal for an amino acid.

3.9. Total polyphenol contents of mealworms in different metamorphic stages

The TPC of mealworms varied significantly with the metamorphic stages ($P = 0.01$; Fig. 5). More specifically, adults ($P = 0.029$) and pupae ($P = 0.01$) had significantly higher TPC than larvae, whereas the adults and pupae had similar TPC levels.

3.10. In vitro digestibility of mealworms at different metamorphic stages

The *in vitro* DM ($P < 0.0001$ for both) and CP ($P < 0.0001$ for both) digestibility of mealworms for both monogastric and ruminant animals was affected by the metamorphic stage of the mealworm (Fig. 6). For monogastric animals, mealworm adults had significantly lower DM ($<65\%$) and CP ($<60\%$) *in vitro* digestibility compared to larvae (DM: $P = 0.0004$; CP: $P = 0.0002$), pupae (DM: $P = 0.0004$; CP: $P = 0.0002$), and SBM (DM: $P = 0.0006$; CP: $P = 0.0001$) whereas the DM and CP

Table 8

The chemical scores of essential amino acids present in different metamorphic stages of the mealworm raised under a wheat-based substrate.

Amino acids	Larvae	Pupae	Adults	SBM	P value
Phe	205 ± 4.4 ^b	208 ± 5.2 ^b	164 ± 4.4 ^c	240 ± 8.3 ^a	<0.0001
Val	148 ± 6.5 ^a	145 ± 7.6 ^a	158 ± 6.5 ^a	104 ± 12.1 ^b	0.0099
Trp	124 ± 3.2 ^b	156 ± 3.8 ^a	147 ± 3.2 ^a	146 ± 6.0 ^a	<0.0001
Thr	138 ± 1.0 ^a	139 ± 1.1 ^a	123 ± 1.0 ^b	118 ± 1.8 ^b	<0.0001
Ile	177 ± 1.9 ^a	173 ± 2.3 ^a	177 ± 1.9 ^a	156 ± 3.6 ^b	<0.0001
Met	99 ± 3.8 ^a	99 ± 4.5 ^{ab}	83.1 ± 3.8 ^b	88 ± 7.2 ^{ab}	0.032
Met + Cys	81 ± 1.7 ^a	76 ± 2.1 ^a	67 ± 1.7 ^b	85 ± 3.3 ^a	<0.0001
His	253 ± 2.5 ^b	247 ± 3.0 ^b	278 ± 2.5 ^a	166 ± 4.7 ^c	<0.0001
Arg	346 ± 22.5	336 ± 26.6	287 ± 22.5	350 ± 42.0	0.267
Leu	162 ± 1.5 ^b	161 ± 1.7 ^b	174 ± 1.5 ^a	145 ± 2.8 ^c	<0.0001
Lys	137 ± 3.5 ^a	130 ± 4.1 ^a	103 ± 3.5 ^b	117 ± 6.5 ^{ab}	<0.0001

Results are presented as mean ± standard errors of the mean. Treatment groups with different letters in the superscripts within a row are significantly different ($P < 0.05$). SBM, soybean meal; Phe, phenylalanine; Val, valine; Thr, Trp, tryptophan; threonine; Ile, isoleucine; Met, methionine; Cys, cysteine; His, histidine; Arg, arginine; Leu, leucine; Lys, lysine.

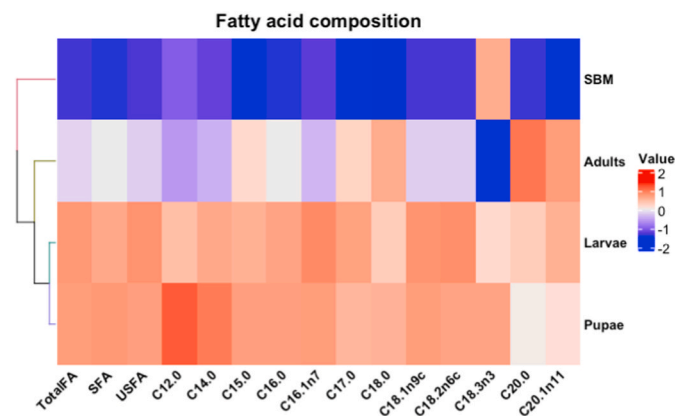


Fig. 4. Heatmap of fatty acid composition among different metamorphic stages of mealworm larvae compared to those in soybean meal (SBM). Fatty acid compositions are clustered into three distinct clusters: 1) SBM, 2) Adults, and 3) Larvae and Pupae. Larvae and pupae are closely linked to each other in terms of their fatty acid profiles.

digestibility of larvae and pupae, and SBM were similarly high (>90%). For ruminants, the *in vitro* digestibility for both DM and CP followed a trend similar to that of monogastric animals. The DM (<65%) and CP (<55%) digestibility for mealworm beetles were lower compared to the values for larvae ($P < 0.001$ for both DM and CP), pupae ($P < 0.001$ for both DM and CP), and SBM ($P < 0.001$ for both DM and CP). In addition, the *in vitro* CP (but not DM) digestibility of SBM was significantly higher compared to the values for larvae ($P = 0.005$) and pupae ($P = 0.007$), whereas values in pupae and larvae did not differ.

3.11. Mealworm larval gut microbiome

The gut microbiome of larvae was found to be dominated by Firmicutes and Proteobacteria (Suppl. Figures 3 and 4). Firmicutes was the most dominant phylum in both fasted and fed larvae, the relative abundance of which decreased when exposed to fasting (Fig. 7). Interestingly, the abundance of Proteobacteria increased during food

Table 9

The fatty acid profile (g/kg dry matter, DM) of different metamorphic stages of the mealworm raised under a wheat-based substrate.

Fatty acids	Larvae	Pupae	Adults	SBM	P value
C12:0	0.646 ± 0.0435 ^b	0.976 ± 0.0515 ^a	0.187 ± 0.0435 ^c	0.0 ± 0.0814 ^c	<0.0001
C14:0	11.88 ± 0.662 ^a	14.03 ± 0.783 ^a	5.07 ± 0.662 ^b	0.03 ± 1.239 ^c	<0.0001
C15:0	0.307 ± 0.0140 ^{ab}	0.33 ± 0.0166 ^a	0.251 ± 0.0140 ^b	0.015 ± 0.0263 ^c	<0.0001
C16:0	43.16 ± 2.01 ^a	43.86 ± 2.37 ^a	30.60 ± 2.01 ^b	3.58 ± 3.75 ^c	<0.0001
C16:1n7	6.69 ± 0.328 ^a	6.27 ± 0.388 ^a	2.75 ± 0.328 ^b	0.04 ± 0.613 ^c	<0.0001
C17:0	0.489 ± 0.0464 ^a	0.452 ± 0.0549 ^a	0.393 ± 0.0464 ^a	0.030 ± 0.0868 ^b	0.001987
C18:0	7.80 ± 0.439 ^a	8.74 ± 0.519 ^a	9.01 ± 0.439 ^a	0.72 ± 0.821 ^b	<0.0001
C18:1n9c	139.10 ± 4.88 ^a	134.43 ± 5.78 ^a	74.59 ± 4.88 ^b	3.36 ± 8.93 ^c	<0.0001
C18:2n6c	71.9 ± 2.00 ^a	67.2 ± 2.32 ^a	42.3 ± 10.8 ^b	10.8 ± 3.67 ^c	<0.0001
C18:3n3	1.230 ± 0.121 ^a	1.398 ± 0.143 ^a	0.679 ± 0.12 ^b	1.370 ± 0.227 ^a	0.0044
C20:0	0.243 ± 0.0237 ^{ab}	0.210 ± 0.0281 ^b	0.324 ± 0.0237 ^a	0.050 ± 0.044 ^c	0.0003546
C20:1n11	0.241 ± 0.0211 ^a	0.200 ± 0.0249 ^a	0.259 ± 0.0211 ^a	0.045 ± 0.0394 ^b	0.001325
SFA	64.53 ± 2.61 ^a	68.60 ± 3.08 ^a	45.84 ± 2.61 ^b	4.43 ± 4.87 ^c	<0.0001
USFA	219.1 ± 6.97 ^a	209.5 ± 8.25 ^a	120.6 ± 6.97 ^b	15.6 ± 13.04 ^c	<0.0001
TFA	284 ± 8.99 ^a	278 ± 10.63 ^a	166 ± 8.99 ^b	20.1 ± 16.81 ^c	<0.0001

Results are presented as mean ± standard errors of the mean. Treatment groups with different letters in the superscripts within a row are significantly different ($P < 0.05$). SBM; C12:0, lauric acid; C14:0, myristic acid; C15:0, pentadecylic acid; C16:0, palmitic acid; C16:1n7, palmitoleic acid; C17:0, margaric acid; C18:0, stearic acid; C18:1n9c, oleic acid; C18:2n6c, linoleic acid; C18:3n3, alpha-linolenic acid; C20:0, behenic acid; C20:1n11, gadoleic acid; SFA, saturated fatty acids; USFA, unsaturated fatty acids; TFA, total fatty acids.

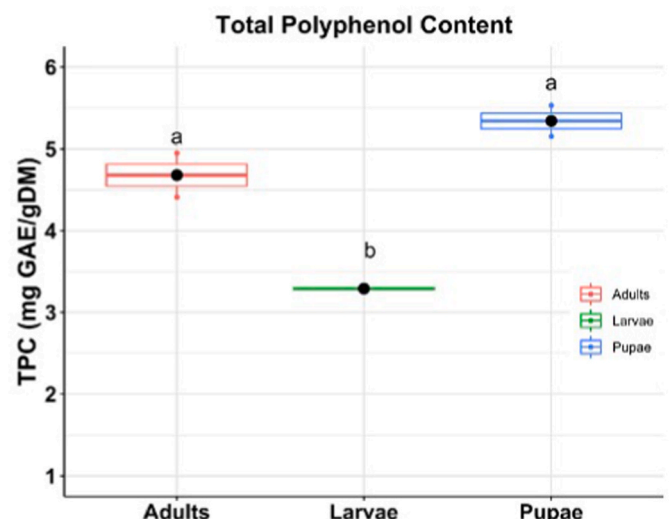


Fig. 5. Total polyphenol contents among different developmental stages of mealworm, expressed as mg gallic acid equivalents per g dry matter.

deprivation (Fig. 7A). Although the species richness showed a higher median value under fasted conditions compared to the fed larvae, the effect was insignificant (Fig. 7B). The structure of the larval gut microbiome differed in response to a short-term nutritional challenge (fed vs.

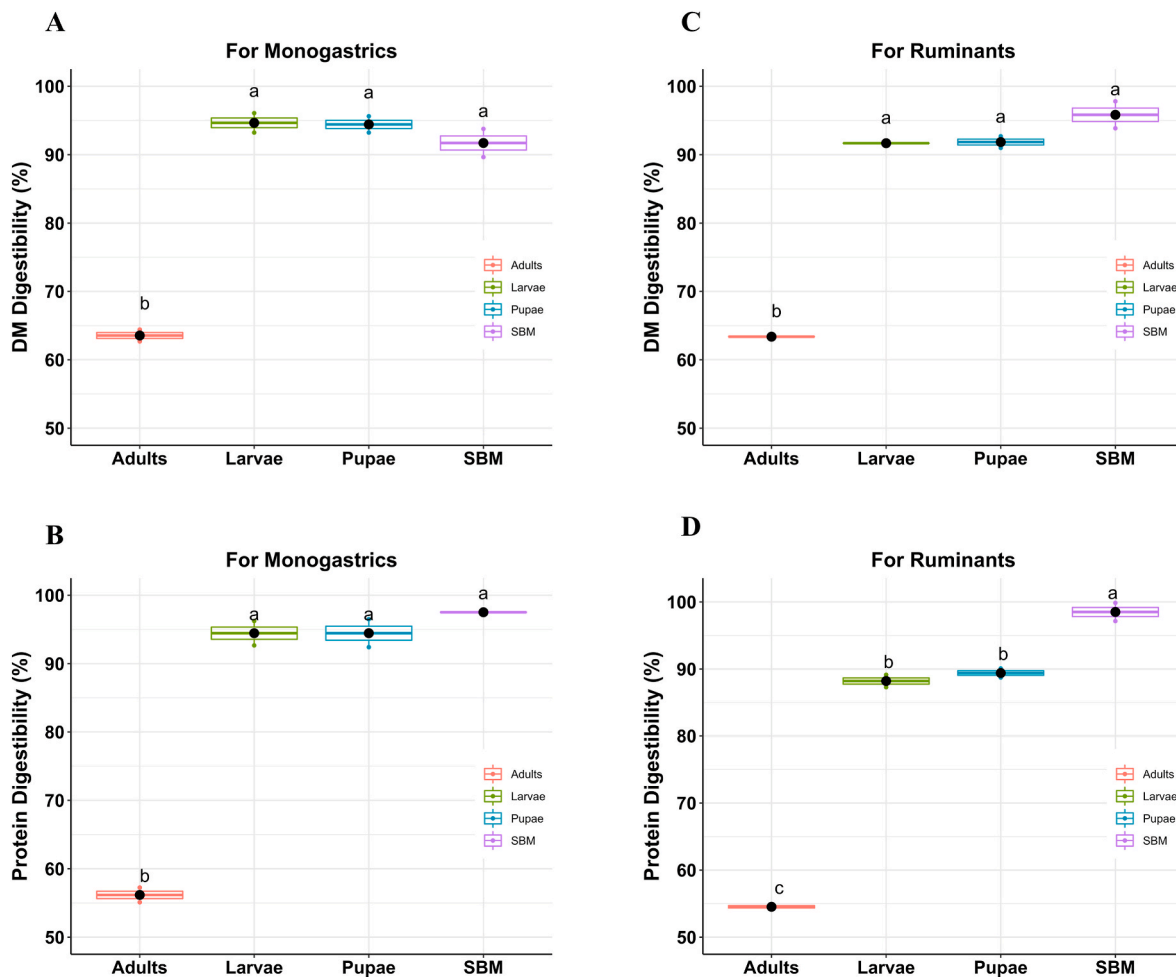


Fig. 6. *In vitro* digestibilities of dry matter (DM) and protein simulated for monogastric and ruminant animals. A. *In vitro* DM digestibilities of mealworm adults, larvae, and pupae, and soybean (SBM) for monogastric animals, B. *In vitro* protein digestibility of mealworm adults, larvae, and pupae, and SBM for monogastric animals. C. *In vitro* DM digestibility of mealworm adults, larvae, and pupae, and SBM for ruminant animals, D. *In vitro* protein digestibility of mealworm adults, larvae, and pupae, and SBM for ruminant animals.

fasted situation). The PCoA ordination plot showed a separation in the community structure of the microbiome in fed and fasted larvae inferring a driving effect of food availability in the larval gut microbiome (Fig. 8). PERMANOVA showed significant differences in bacterial communities between the fed and fasted groups (Adonis, $R^2 = 0.11$, $P < 0.06$). The differential abundance analysis of bacterial ASV also showed a distinct enrichment of bacterial taxa in fasted larvae compared to limited taxa in fed larvae (Fig. 9). Although *Clostridium* was enriched in the fed larvae, *Brevibacillus*, *Aeribacillus*, *Anoxybacillus*, and *Variovorax* were among the taxa most enriched in the fasted larvae (Fig. 9).

4. Discussion

The present study hypothesized that a) yellow mealworms (*Tenebrio molitor*) of high nutritional quality can be produced in the Nordic region using locally available agricultural by-products, b) the metamorphic stage of mealworms affects their nutritional values, and potential utilization as animal feed ingredients, and c) the gut microbiome profile of the mealworm larvae is altered in response to short-term nutritional challenges. The study highlights that mealworms with high nutritional properties, similar to those of a commercial SBM, can be produced using locally available grain-based by-products from the Nordic region. The metamorphic stage of mealworms was shown to affect their chemical composition and nutritional values, with larvae and pupae generally sharing a common nutritional profile. The present study also identified a

unique and rather simple set of microbes in the gut microbiome of mealworm larvae and showed that a nutrient-deficient situation markedly altered the larval gut microbial communities. These findings provide a comprehensive and empirical understanding of the nutritional profile of the yellow mealworm in different stages of development and insights into the mealworm larval gut microbiome, which are critical for further studies aiming to identify and utilize insect-based alternative protein sources.

4.1. Nutritional profiles of yellow mealworm larvae produced using local agricultural by-products from the Nordic region

Insects can play a principal role in the sustainable recycling of low-grade bioresources, including agricultural by-products (Adhikari et al., 2021). The present study demonstrates that mealworms can effectively utilize grain-based (wheat or oat) by-products from the Nordic region. An earlier study also suggested that agricultural by-products, including wheat-based resources, could be used to achieve an acceptable growth performance of mealworm larvae (Zhang et al., 2019). The chemical and nutritional characterization of mealworm rearing substrates used in this study generally revealed a different mineral profile as evidenced by higher total ash, Mg (>2-fold), K (>1.7-fold), Fe, and Zn (>1.6-fold) and lower Ca levels in WB than in OB. Such differences in mineral contents of rearing substrates were reflected in the larvae with higher total ash, Mg, and Zn and lower Ca levels in WB-fed larvae compared to those fed OB. A

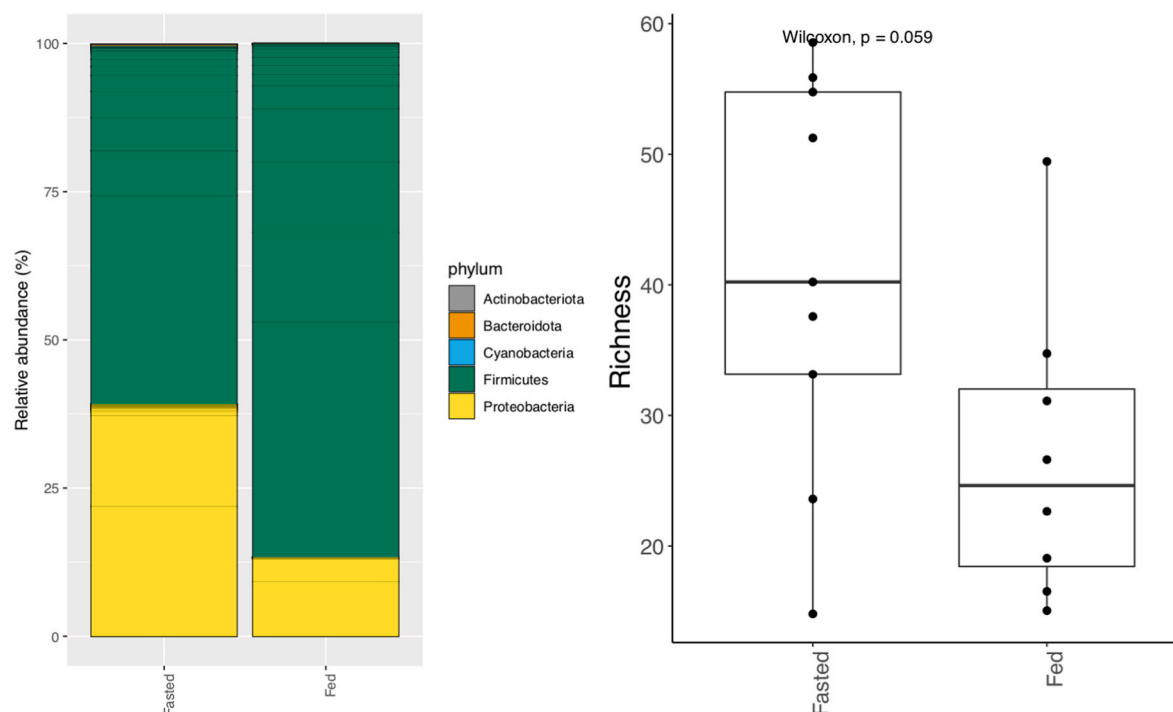


Fig. 7. The relative abundance and richness of bacterial microbiome in the mealworm larval gut. A) Comparative bar chart showing the relative abundance of gut bacteria belonging to different phyla in fasted and fed mealworm larvae. B) Box plots show observed diversity (richness) of bacterial amplicon sequence variants (ASVs) in fed and fasting treatments.

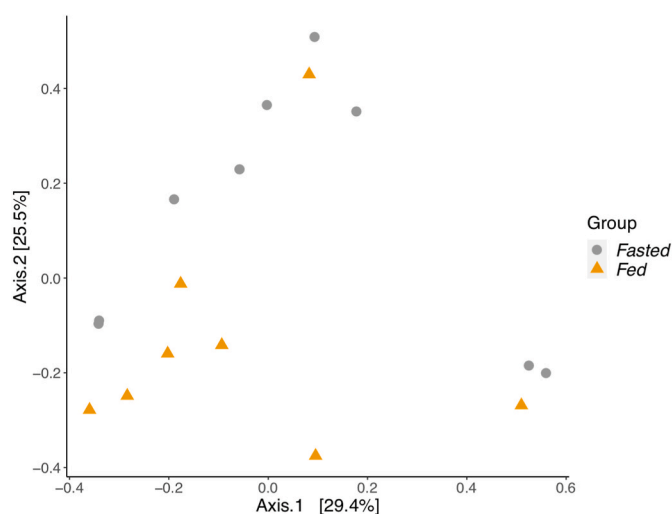


Fig. 8. Principal coordinates analysis (PCoA) based on Bray-Curtis distance matrices of communities in the larval gut of yellow mealworm. The first and second principal coordinates are based on the gut microbiome composition in fasted and fed larvae.

similar pattern was also observed for fat, with higher fat content (~ 32 vs. ~ 27 %DM) in the larvae fed WB compared to those provided with OB. Previous studies also indicated that the fat content in mealworm species could vary between dietary substrates with organic by-products (Van Broekhoven et al., 2015). Thus, it suggests that the nutritional values of mealworm larvae, particularly minerals and fat, can be improved through relevant dietary substrates. This further points out the importance of rearing substrates on the nutritional values of mealworms and could compensate for the existing gap of the lower mineral content of mealworms compared to other insect species of a commercial production potential, such as black soldier flies and house flies (Veldkamp

and Bosch, 2015). On the other hand, yellow mealworms are considered the highest fat-containing species among edible insects (Paul et al., 2017). The mealworm larvae in this study contained up to $\sim 32\%$ fat (DM basis), rich in unsaturated fatty acids ($>75\%$ of total fatty acids), and thus they could be used as a vital source of fat in addition to protein.

The protein content of dietary sources can also positively influence the protein level in mealworm larvae (Rumbos et al., 2020). However, we could not detect such a difference in protein levels between larvae-fed OB ($\sim 42\%$ CP, DM) and WB ($\sim 40\%$ CP, DM) in this study, which might be due to the only marginal difference in the protein content of OB and WB (12% vs. 14% CP, DM). Furthermore, an earlier study indicates that protein-rich diets could be essential for mealworm larval growth and survival, but they do not necessarily improve the larval protein content (Van Broekhoven et al., 2015). The nutritional profiles of the mealworm larvae observed in this study corroborated earlier findings (Table 10). Mealworm larvae fed wheat or rye barn had 44–48% CP and 26–30% fat (Bordiean et al., 2022), whereas wheat-barn-fed mealworm larvae contained 37% CP and 44% fat (Zielińska et al., 2021). Additionally, mealworm larvae or pupae exposed to semolina, flour, and oat flakes had $\sim 44\%$ CP, and $\sim 38\%$ fat (Toviho and Bársony, 2022), and mealworm larvae reared on multiple agro-by-products contained 46–52% protein and 27–34% fat (Montalbán et al., 2022). Mealworm larvae exposed to carob meal contained 45% protein and 24% fat (Antonopoulou et al., 2022). Results from these earlier studies agree with our findings, with minor differences in the larval protein content. Such differences in the larval protein levels may be associated with rearing substrates. In addition, it is noteworthy that the use of different nitrogen-to-protein conversion factors, 4.76 in this study vs. standard 6.25 (Zhao et al., 2016; Zielińska et al., 2015) or 5.41 (Bordiean et al., 2022) may have contributed to variations in the larval protein contents across studies. Overall, this study suggests that by-products based on oat and wheat can be successfully used to produce yellow mealworm larvae with an excellent nutritional profile ($>40\%$ CP and $\sim 30\%$ fat, DM). Wheat-based bioresources may serve as a slightly superior rearing substrate than oat-based by-products. Regarding

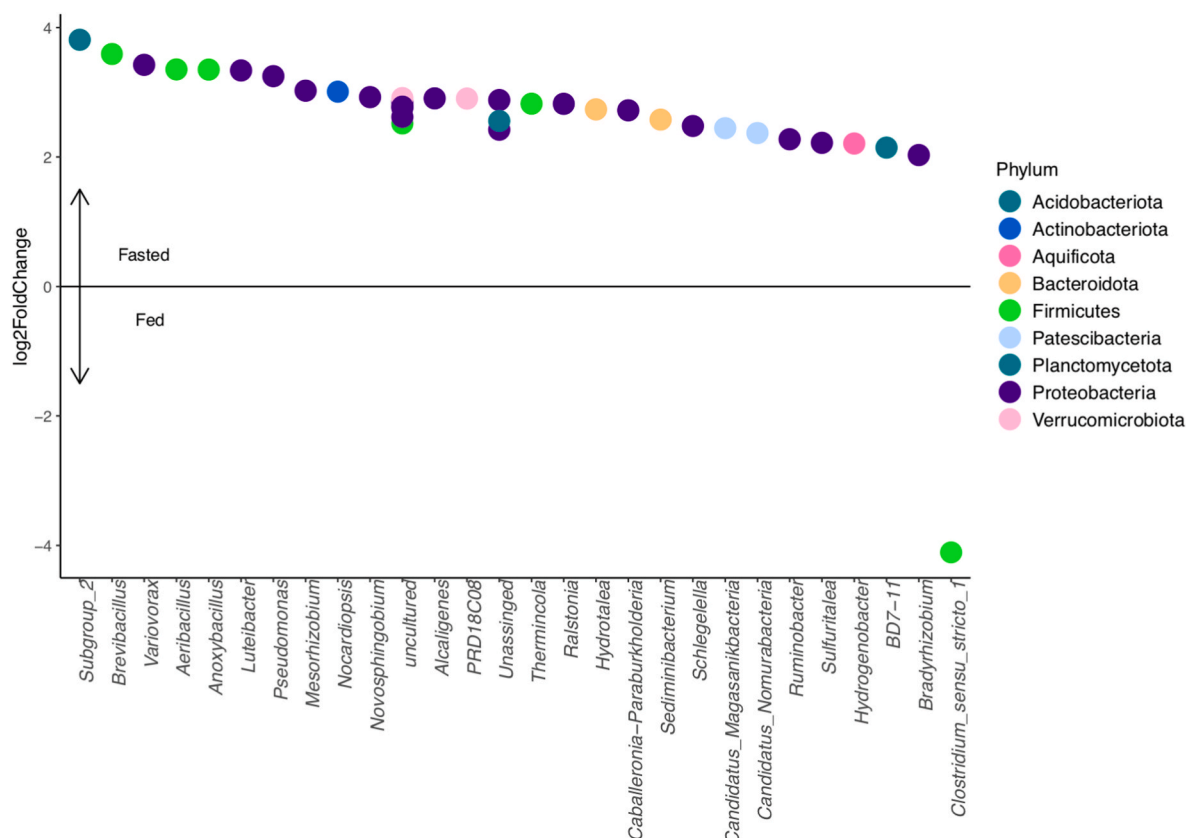


Fig. 9. Differential abundance analysis showing enrichment of bacterial genera in the gut of fasted and fed mealworm larvae. The color of each genus relates to the phylum. The significance of bacterial abundance was determined using the Wald Significance test ($P < 0.05$).

Table 10

Comparison of nutritional values of different mealworms (Coleoptera: Tenebrionidae) reared under diverse feeding substrates or by-products.

Common name	Zoological name	Stage	Rearing substrates	Protein (%DM)	Fat (%DM)	References
YMW	<i>Tenebrio molitor</i>	Larvae	Oat/wheat by-products	40–42 ^a	27–32	This study
YMW	<i>Tenebrio molitor</i>	Pupae	Oat/wheat by-products	40 ^a	29	This study
YMW	<i>Tenebrio molitor</i>	Pupae	Semolina, flour, and oat flakes	44 ^{NS}	38	Toviho and Bársony (2022)
YMW	<i>Tenebrio molitor</i>	Adults	Oat/wheat by-products	47 ^a	18	This study
YMW	<i>Tenebrio molitor</i>	Larvae	Wheat/rye bran	44–48 ^b	26–30	Bordiean et al. (2022)
YMW	<i>Tenebrio molitor</i>	Larvae	Wheat bran	37 ^a	44	Zielińska et al. (2021)
YMW	<i>Tenebrio molitor</i>	Larvae	Multiple agro-by-products	46–52 ^{NS}	27–34	Montalbán et al. (2022)
YMW	<i>Tenebrio molitor</i>	Larvae	Semolina, flour, and oat flakes	43–45 ^{NS}	38–39	Toviho and Bársony (2022)
YMW	<i>Tenebrio molitor</i>	Larvae	Carob meal	45 ^a	24	Antonopoulou et al. (2022)
SW	<i>Zophobas morio</i>	Larvae	Wheat bran	48–49 ^a	31–34	Zielińska et al. (2021); Kulma et al. (2020)
SW	<i>Zophobas morio</i>	Larvae	Wheat, corn, and soybean meal	47 ^c	45	Araújo et al. (2019)
LMW	<i>Alphitobius diaperinus</i>	Larvae	Wheat bran and chicken feed (4:1)	64 ^c	24	Kurečka et al. (2021)
LMW	<i>Alphitobius diaperinus</i>	Pupae	wheat bran and chicken feed (4:1)	71 ^c	19	Kurečka et al. (2021)

YMW, yellow mealworm; SW, superworm; LMW, lesser mealworm.

^{NS} Nitrogen-to-protein conversion factor = Not specified.

^a Nitrogen-to-protein conversion factor = 4.76.

^b Nitrogen-to-protein conversion factor = 5.41.

^c Nitrogen-to-protein conversion factor = 6.25.

comparing the nutritional profiles of yellow mealworms with other mealworms (Table 10), superworm (*Zophobas morio*) larvae fed wheat, corn, and soybean meal had similar protein and higher fat (47% CP, 45% fat) than the contents in yellow mealworm larvae (Araújo et al., 2019). The nutritional compositions of lesser mealworm (*Alphitobius diaperinus*) larvae fed wheat bran and chicken feed presented higher protein but similar or lower fat (64% CP, 24% fat) than those found in yellow mealworm larvae (Kurečka et al., 2021). Such variations in the nutritional composition among mealworms could be attributed to diverse rearing substrates, different nitrogen-to-protein conversion factors, or mealworm species-specific differences.

Insect meals have been characterized as a renewable, alternative, and sustainable source of nutrients for production animals (Sánchez-Muros et al., 2014). Our comparative assessment of the nutrient profile in yellow mealworm larvae and a commercial SBM suggests that mealworm larvae can serve as an alternative nutrient source in the future since *T. molitor* represents one of the highest protein-containing coleopterans (Rumpold and Schlüter, 2013). For production animals, particularly monogastrics, the quality of protein, such as the amino acid profile, in the feed is vital for their optimal growth and productivity (Zhang et al., 2021). This study exhibits that mealworm larvae can serve as an alternative protein source to

monogastric animals, as evidenced by similar essential amino acid levels and higher EAAI (for growing piglets) than those of SBM. The amino acid content can be even higher in mealworm protein isolates than in soybean protein isolates (Yu et al., 2021). In addition to total essential amino acids, individual essential amino acids were comparable in mealworm larvae and SBM, except for two non-limiting amino acids: Phe and Trp were higher in SBM, whereas His was higher in larvae. In agreement with previous studies (Veldkamp and Bosch, 2015), both mealworm larvae and SBM had similar limiting amino acids for growing pigs: Met or Met + Cys. However, CS for Met was even higher in larvae than in SBM (~99 vs. ~88), suggesting that mealworm larvae represent an equally good source as feed ingredients, if not superior, than a commercial SBM in terms of fulfilling amino acids requirements for monogastric animals.

Digestibility is crucial when evaluating new feed ingredients, as it is directly associated with animal growth performance (Liu et al., 2013; Lee et al., 2022). This study revealed that mealworm larvae possess impressive *in vitro* digestibility characteristics (~90%) for both DM and CP. Previous *in vivo* studies also suggest that mealworm larvae-based feed had no negative influences on feed intake and growth of broilers and resulted in a better feed conversion ratio than a SBM-based diet (Bovera et al., 2015). Similarly, a 10% inclusion of mealworm larvae in the diet of growing pigs improved the digestibility of nutrients, including different essential or non-essential amino acids (Yoo et al., 2019). To the best of our knowledge, this study has, for the first time, characterized *in vitro* DM and CP digestibility of mealworms for ruminants. The DM digestibility was similar (>90%) to SBM, but slightly lower rumen digestibility of CP in mealworm larvae than in SBM might be associated with the inhibition of ruminal fermentation due to the high fat content in the mealworm larvae (Palmquist, 1994) or adverse impacts on specific microbes associated with protein digestion. However, the high unsaturated fat and fatty acids content of mealworm larvae may contribute to reduce enteric CH₄ emissions. Indeed, the inclusion of insect oils (5% DM) reduced CH₄ production without altering volatile fatty acid composition in a previous *in vitro* rumen fermentation study (Jayanegara et al., 2020). All this evidence suggests that mealworm larvae produced locally using crop-based agricultural by-products in the Nordics could serve as a promising alternative feed ingredient for both monogastric and ruminant animals. This is critical because protein self-sufficiency for animal feed production is low in Europe, including the Nordic region (de Visser et al., 2014, 2014b; Åby et al., 2014), and the future supply of soybeans from South America is not sustainable as it comes with high carbon emissions and energy cost (da Silva et al., 2010; Macedo et al., 2012; Dreoni et al., 2021).

4.2. Yellow mealworm nutritional profile is dependent upon its metamorphic stage

Economic interest in commercial insect farming is growing in Europe with increasing investments in production technologies and automation (Thrustardottir et al., 2021; Niyonsaba et al., 2021). Larvae would undoubtedly be a preferred metamorphic stage of yellow mealworm production industries, and in fact, several studies have highlighted the importance of mealworm larvae as a feed source (Veldkamp and Bosch, 2015; Zhang et al., 2019). However, commercial insect production, including mealworms, can generate a significant amount of biomass related to other metamorphic stages, such as pupae and adults, during breeding, colony maintenance, and overall management of production activities. Chemical or nutritional parameters in other metamorphic stages of mealworms than larvae or related by-products, such as exuviae, are largely unknown (Ravzanaadii et al., 2012). To our knowledge, this is the first study to evaluate the comprehensive chemical, nutritional and digestibility characteristics of yellow mealworms at their different metamorphic stages.

The current study showed that mealworm larvae and pupae have common nutritional attributes, as evidenced by similar protein, fat,

energy contents, amino and fatty acid profiles, and DM and protein digestibility simulating both monogastric and ruminant animals. Both larvae and pupae had similar essential amino acid content and had a significantly high proportion of unsaturated fatty acids (~75%). These nutritional characteristics agree with previous reports (Ravzanaadii et al., 2012). The only prominent differences were that the larvae had high fiber and mineral contents and low polyphenols levels than the pupae. Higher polyphenols in pupae (and adults) compared to larvae could be associated with the development of the exoskeleton, as polyphenols are considered an essential component in cuticle hardening in insects (Wigglesworth, 1988). The adult stage of mealworms demonstrated unique chemical and nutritional properties with higher protein, polyphenol, and CF (>3-fold) and lower NFE and fat contents than the levels in larvae or pupae. The greater CP content in adults could partly be attributed to the formation of non-soluble nitrogenous compounds containing tissues like cuticle and structural polysaccharides (Moran, 1959), but both essential and non-essential amino acids contents were greater in adults than in larvae, pupae, or SBM. The high CF fraction observed in adults could be attributed to chitin, one of the most abundant polysaccharides that consists of the β -1,4-linkage of N-acetyl-D-glucosamine (Janssen et al., 2017; Wysokowski et al., 2015). Poor *in vitro* digestibility (<30%) of mealworm adults than in larvae, pupae or SBM in this study could be associated with their high chitin-related substances (Shin et al., 2019) as the inclusion of chitin in diets was shown to adversely affect nutrient digestibility in broilers (Khempaka et al., 2011). Thus, suitable animal nutrition strategies, such as using enzymes or selecting animal species with a high chitinase activity in the gut (Tabata et al., 2018), are necessary while aiming to use mealworm adults as animal feed ingredients.

The inclusion of adults or chitin in the animal diets could, however, lead to antimicrobial properties as lower cecal short-chain fatty acid contents in broilers were found when fed chitin-based diets (Razdan and Pettersson, 1994). Moreover, it also led to favorable metabolic changes in broilers, such as low plasma cholesterol levels and reduced liver triglyceride contents (Razdan and Pettersson, 1994; Hossain and Blair, 2007). In young pigs, chitoooligosaccharides were shown to improve feed efficiency and inhibit the growth of harmful gut microbiota (Han et al., 2007). It is not known whether whole insects or insect-derived chitin-related compounds possess health-promoting properties in ruminants or have specific anti-methanogenic characteristics inhibiting the growth or activity of rumen methanogenic microbes. Nevertheless, the present study indicates that mealworm larvae and pupae, with similar nutritional profiles, could be alternative nutrient sources for production animals with high digestibility values. Allowing larvae to develop into the pupae may not necessarily lead to a significant comparative advantage toward higher nutritional values. Adult mealworms could be a vital source of antimicrobial and bioactive compounds, which could positively influence animal health and immune function; however, ways to improve their digestibility must be identified. Considering the nutritional value and production potential, mealworm larvae could be a suitable option to use as animal feed ingredients. Efficient recycling of a diverse range of bioresources by applying novel approaches, such as understanding and modulation of gut microbiome profile, would be beneficial for future mealworm industries, as discussed below.

4.3. The gut microbiome of mealworm larvae possesses a unique and rather simple set of microbes

The gut microbial communities have been thoroughly explored in several phylogenetically diverse mammalian species (Nishida and Ochman, 2018), elucidating their role in the adaptation to environmental changes or highlighting a specific relationship with the host (McKenzie et al., 2017). The gut microbiota in *T. molitor* can also facilitate adaptation in terms of their composition and abundance in response to environmental factors (Cambon et al., 2018), including changes in the dietary composition (Lou et al., 2021). This study

provided new knowledge on how the gut microbiome of mealworm larvae is modulated in response to immediate nutritional challenges, as short-term starvation (gut emptying) is a standard step in the post-harvesting handling of insects (Wynants et al., 2017). The gut microbiome in mealworm larvae under a WB substrate in this study was mainly dominated by Firmicutes and Proteobacteria phyla, and a similar microbiome structure for mealworm larvae has also been previously reported (Brandon et al., 2018; Przemieniecki et al., 2020). At the genus level, *Spiroplasma*, *Lactococcus*, *Ralstonia*, *Tyzzera*, and *Enterococcus*, among others, were the primary group of microbial communities present in the larval gut, as previously indicated (Lou et al., 2021). This study suggests that the digestive tract of mealworm larvae may possess a rather simple microbiome structure that harbors limited microbial communities.

Interestingly, overall gut microbial richness was found to be higher in fasted situations, as several microbial species of Firmicutes and Proteobacteria were highly enriched in response to starvation. This infers that the gut microbiome of mealworm larvae is highly plastic and responds quickly, even to short-term nutritional challenges. In particular, *Brevibacillus* was increased in fasting situations, and certain *Brevibacillus* strains in insects are associated with broad-spectrum antimicrobial activity against, e.g., phytopathogenic bacteria or fungi (Ruiu, 2013). Additionally, an increased abundance of *Ralstonia* and *Bacillus* in the fasted larval gut could be associated with efficient nutrient utilization since they are recognized as sugar-fermenting bacteria (Rajagopal, 2009). There is a clustering of bacterial communities under fasted and fed conditions, indicating the strong adaptability of microbes to the changing environment (Brandon et al., 2018). However, it was also observed a few larvae samples considered fed clustered closer to the fasted samples. This could be due to the inability of individual larvae within that feeding group to be adequately fed on the feeding tray at the time of sampling. Overall, changes in the dynamics of Firmicutes and Proteobacteria phyla during starvation demonstrate their associations with the nutritional status of larvae; however, future studies are needed to identify the microbes at the species level. Such a strong adaptation potential of the gut microbiome, even during short-term nutritional challenges, could be exploited in the future to improve the efficiency of bioresource recycling in commercial mealworm production settings (Adhikari et al., 2021).

In the present study, only microbes enriched in fed conditions were from the *Clostridium* genus (Firmicutes). Our findings agree with a previous study that reported that gut microbial diversity of mealworm larvae raised under wheat barn as a rearing substrate was low, but a high abundance of microbes belonging to *Clostridium* were found along with *Enterococcus* and *Erwinia* (Ong et al., 2018). *Clostridium* appears to play a central role in the feed degradation in mealworm larvae (Przemieniecki et al., 2020). Our study points out that suboptimal nutrient availability in the digestive tract can play a vital role in modulating the gut microbiome in mealworm larvae. The primary enrichment in several microbial communities in the larval gut during suboptimal nutritional situations is perhaps associated with survival, nutrition, and specific immune responses (Engel and Moran, 2013). The present study indicates that the gut microbiome could potentially be exploited as a novel tool to improve the efficiency of bioresource recycling by insect larvae.

5. Conclusions

Sustainable livestock production must be established through the identification and use of alternative feed ingredients to meet global food demands in the context of the rising global population and the dietary shift toward animal-based protein. Based on the data on nutritional composition and *in vitro* digestibility, yellow mealworm larvae could be a critical alternative protein source to replace commercially used soybean-based protein. Furthermore, mealworm pupae and larvae have a similar nutritional profile, and the pupal stage may not necessarily provide additional benefits to larvae in terms of nutritional composition

and nutrient utilization. Mealworm adults could be an important source of bioactive compounds with beneficial effects on animal health and immune function, but approaches to improve their digestibility and potential utilization must be identified. In addition, the potential impact of mealworms on rumen microbial communities and subsequent beneficial effects on enteric CH₄ emissions should be further evaluated. Furthermore, it was revealed that Firmicutes and Proteobacteria dominate the gut microbiome of mealworm larvae, and several genera of them are enriched during starvation. On the other hand, the *Clostridium* genus was promoted when exposed to a WB feed. Future studies are needed to evaluate whether the mealworm larval gut microbiome serves as a unique tool to improve the efficiency of bioresource recycling and the nutritional values of larvae. Nevertheless, the present study provides a strong platform for further commercialization of the future mealworm production sector, exploiting mealworm biomass derived from different metamorphic stages.

CRedit authorship contribution statement

Prabhat Khanal: Conceptualization, Methodology, Software, Data curation, Formal analysis, Investigation, Funding acquisition, Resources, Project administration, Writing – original draft, Writing – review & editing. **Deepak Pandey:** Methodology, Data curation, Formal analysis, Writing – review & editing. **Geir Næss:** Methodology, Project administration, Writing – review & editing. **Ana R.J. Cabrita:** Methodology, Data curation, Resources, Writing – review & editing. **António J.M. Fonseca:** Methodology, Data curation, Resources, Writing – review & editing. **Margarida R.G. Maia:** Methodology, Data curation, Resources, Writing – review & editing. **Bishnu Timilisina:** Data curation, Formal analysis, Writing – review & editing. **Teun Veldkamp:** Methodology, Data curation, Writing – review & editing. **Rumakanta Sapkota:** Methodology, Data curation, Software, Resources, Writing – review & editing. **Hege Overrein:** Methodology, Data curation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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References

- Åby, B., Kantanen, J., Aass, L., et al., 2014. Current status of livestock production in the Nordic countries and future challenges with a changing climate and human population growth. *Acta Agriculturae Scandinavica, Section A—Animal Science* 64 (2), 73–97.
- Adhikari, P., Aryal, N., Ghimire, A., et al., 2021. Sustainable Biowaste Recycling Using Insects. *Clean Energy and Resources Recovery*. Elsevier, pp. 399–420.
- Alexandratos, N., Bruinsma, J., 2012. *World Agriculture towards 2030/2050: the 2012 Revision*.
- Antonopoulou, E., Panteli, N., Feidantsis, K., et al., 2022. Carob (*Ceratonia siliqua*) as functional feed is beneficial in yellow mealworm (*Tenebrio molitor*) rearing: evidence from growth, antioxidant status and cellular responses. *Antioxidants* 11 (9), 1840.
- AOAC. Association of Official Agricultural Chemists, 2001. AOAC Official Method 2001.11 Protein (Crude) in Animal Feed, Forage (Plant Tissue), Grain, and Oilseeds Block Digestion Method Using Copper Catalyst and Steam Distillation into Boric Acid.
- Araújo, R.R.S., dos Santos Benfina, T.A.R., Ferraz, V.P., et al., 2019. Nutritional composition of insects *Gryllus assimilis* and *Zophobas morio*: potential foods harvested in Brazil. *J. Food Compos. Anal.* 76, 22–26.
- Biasato, I., Gasco, L., De Marco, M., et al., 2017. Effects of yellow mealworm larvae (*Tenebrio molitor*) inclusion in diets for female broiler chickens: implications for animal health and gut histology. *Anim. Feed Sci. Technol.* 234, 253–263.
- Boisen, S., Fernández, J., 1997. Prediction of the total tract digestibility of energy in feedstuffs and pig diets by in vitro analyses. *Anim. Feed Sci. Technol.* 68 (3–4), 277–286.
- Bolyen, E., Rideout, J.R., Dillon, M.R., et al., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37 (8), 852–857.
- Bordiean, A., Krzyżaniak, M., Aljewicz, M., et al., 2022. Influence of different diets on growth and nutritional composition of yellow mealworm. *Foods* 11 (19), 3075.
- Bovera, F., Piccolo, G., Gasco, L., et al., 2015. Yellow mealworm larvae (*Tenebrio molitor*, L.) as a possible alternative to soybean meal in broiler diets. *Br. Poultry Sci.* 56 (5), 569–575.
- Brandon, A.M., Gao, S.-H., Tian, R., et al., 2018. Biodegradation of polyethylene and plastic mixtures in mealworms (larvae of *Tenebrio molitor*) and effects on the gut microbiome. *Environ. Sci. Technol.* 52 (11), 6526–6533.
- Callahan, B.J., McMurdie, P.J., Rosen, M.J., et al., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13 (7), 581–583.
- Cambon, M., Ogier, J.-C., Lanois, A., et al., 2018. Changes in rearing conditions rapidly modify gut microbiota structure in *Tenebrio molitor* larvae. *bioRxiv*, 423178.
- Caporaso, J.G., Lauber, C.L., Walters, W.A., et al., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. USA* 108 (Suppl. ment 1), 4516–4522.
- Commission, E., 2009. Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. *Off. J. Eur. Union* 54 (1), 2–54.
- da Silva, V.P., van der Werf, H.M., Spies, A., et al., 2010. Variability in environmental impacts of Brazilian soybean according to crop production and transport scenarios. *J. Environ. Manag.* 91 (9), 1831–1839.
- de Visser, C., Schreuder, R., Stoddard, F., 2014. The EU's Dependence on Soya Bean Import for the Animal Feed Industry and Potential for EU Produced Alternatives. *Oilseeds and Fats, Crops and Lipids*.
- Dreoni, I., Matthews, Z., Schaafsma, M., 2021. The impacts of soy production on multi-dimensional well-being and ecosystem services: a systematic review. *J. Clean. Prod.* 130182.
- Eisler, M.C., Lee, M.R., Tarlton, J.F., et al., 2014. Agriculture: steps to sustainable livestock. *Nature* 507 (7490), 32–34.
- Enahoro, D., Lannerstad, M., Pfeifer, C., et al., 2018. Contributions of livestock-derived foods to nutrient supply under changing demand in low-and middle-income countries. *Global Food Secur.* 19, 1–10.
- Engel, P., Moran, N.A., 2013. The gut microbiota of insects—diversity in structure and function. *FEMS Microbiol. Rev.* 37 (5), 699–735.
- Gallili, T., 2015. dendextend: an R package for visualizing, adjusting and comparing trees of hierarchical clustering. *Bioinformatics* 31 (22), 3718–3720.
- Godde, C., Mason-D'Croz, D., Mayberry, D., et al., 2021. Impacts of climate change on the livestock food supply chain; a review of the evidence. *Global Food Secur.* 28, 100488.
- Goering, H.K., Van Soest, P.J., 1970. *Forage Fiber Analyses (Apparatus, Reagents, Procedures, and Some Applications)*. US Agricultural Research Service.
- Gu, Z., Eils, R., Schlesner, M., 2016. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* 32 (18), 2847–2849.
- Han, K., Kwon, I., Lohakare, J., et al., 2007. Chito-oligosaccharides as an alternative to antimicrobials in improving performance, digestibility and microbial ecology of the gut in weanling pigs. *Asian-Australas. J. Anim. Sci.* 20 (4), 556–562.
- Heckmann, L.-H., Andersen, J.L., Gianotten, N., et al., 2018. Sustainable Mealworm Production for Feed and Food. *Edible Insects in Sustainable Food Systems*. Springer, pp. 321–328.
- Hong, J., Han, T., Kim, Y.Y., 2020. Mealworm (*Tenebrio molitor* Larvae) as an alternative protein source for monogastric animal: a review. *Animals* 10 (11), 2068.
- Hossain, S., Blair, R., 2007. Chitin utilisation by broilers and its effect on body composition and blood metabolites. *Br. Poultry Sci.* 48 (1), 33–38.
- Janssen, R.H., Vincken, J.-P., van den Broek, L.A., et al., 2017. Nitrogen-to-protein conversion factors for three edible insects: *Tenebrio molitor*, *Alphitobius diaperinus*, and *Hermetia illucens*. *J. Agric. Food Chem.* 65 (11), 2275–2278.
- Jayanegara, A., Gustanti, R., Ridwan, R., et al., 2020. Fatty acid profiles of some insect oils and their effects on in vitro bovine rumen fermentation and methanogenesis. *Ital. J. Anim. Sci.* 19 (1), 1310–1317.
- Jin, X., Heo, P., Hong, J., et al., 2016. Supplementation of dried mealworm (*Tenebrio molitor* larva) on growth performance, nutrient digestibility and blood profiles in weaning pigs. *Asian-Australas. J. Anim. Sci.* 29 (7), 979.
- Khemphaka, S., Chitsachapong, C., Molee, W., 2011. Effect of chitin and protein constituents in shrimp head meal on growth performance, nutrient digestibility, intestinal microbial populations, volatile fatty acids, and ammonia production in broilers. *J. Appl. Poultry Res.* 20 (1), 1–11.
- Kulma, M., Kourimská, L., Homolková, D., et al., 2020. Effect of developmental stage on the nutritional value of edible insects. A case study with *Blaberus craniifer* and *Zophobas morio*. *J. Food Compos. Anal.* 92, 103570.
- Kurečka, M., Kulma, M., Petříčková, D., et al., 2021. Larvae and pupae of *Alphitobius diaperinus* as promising protein alternatives. *Eur. Food Res. Technol.* 247 (10), 2527–2532.
- Lee, J.-H., Kim, T.-K., Cha, J.Y., et al., 2022. How to develop strategies to use insects as animal feed: digestibility, functionality, safety, and regulation. *J. Anim. Sci. Technol.* 64 (3), 409.
- Liu, S.Y., Selle, P.H., Cowieson, A.J., 2013. Strategies to enhance the performance of pigs and poultry on sorghum-based diets. *Anim. Feed Sci. Technol.* 181 (1–4), 1–14.
- Lou, Y., Li, Y., Lu, B., et al., 2021. Response of the yellow mealworm (*Tenebrio molitor*) gut microbiome to diet shifts during polystyrene and polyethylene biodegradation. *J. Hazard Mater.* 416, 126222.
- Macedo, M.N., DeFries, R.S., Morton, D.C., et al., 2012. Decoupling of deforestation and soy production in the southern Amazon during the late 2000s. *Proc. Natl. Acad. Sci. USA* 109 (4), 1341–1346.
- Marten, G.C., Barnes, R.F., 1979. Prediction of Energy Digestibility of Forages with in Vitro Rumen Fermentation and Fungal Enzyme Systems. Standardization of Analytical Methodology for Feeds: Proceedings. IDRC, Ottawa, ON, CA.
- McDonald, P., Edwards, R.A., Greenhalgh, J.F.D., et al., 2011. *Animal Nutrition*.
- McKenzie, V.J., Song, S.J., Delsuc, F., et al., 2017. The effects of captivity on the mammalian gut microbiome. *Integr. Comp. Biol.* 57 (4), 690–704.
- McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8 (4), e61217.
- Montalbán, A., Sánchez, C.J., Hernández, F., et al., 2022. Effects of agro-industrial byproduct-based diets on the growth performance, digestibility, nutritional and microbiota composition of mealworm (*Tenebrio molitor* L.). *Insects* 13 (4), 323.
- Moran, M.R., 1959. Changes in the distribution of nitrogen during metamorphosis of the mealworm, *Tenebrio molitor* Linnaeus. *J. N. Y. Entomol. Soc.* 67 (3/4), 217–222.
- Nishida, A.H., Ochman, H., 2018. Rates of gut microbiome divergence in mammals. *Mol. Ecol.* 27 (8), 1884–1897.
- Niyonsaba, H., Höhler, J., Kooistra, J., et al., 2021. Profitability of insect farms. *Journal of Insects as Food and Feed* 7 (5), 923–934.
- O'Fallon, J.V., Busboom, J., Nelson, M., et al., 2007. A direct method for fatty acid methyl ester synthesis: application to wet meat tissues, oils, and feedstuffs. *J. Anim. Sci.* 85 (6), 1511–1521.
- Oksanen, J., Blanchet, F., Kindt, R., et al., 2021. Package 'vegan': community ecology package. Version 2 (9), 1–295.
- Ong, S.Y., Kho, H.-P., Riedel, S.L., et al., 2018. An integrative study on biologically recovered polyhydroxyalkanoates (PHAs) and simultaneous assessment of gut microbiome in yellow mealworm. *J. Biotechnol.* 265, 31–39.
- Palmquist, D.L., 1994. The role of dietary fats in efficiency of ruminants. *J. Nutr.* 124 (Suppl. 1.8), 1377S–1382S.
- Pandey, D., Hansen, H.H., Dhakal, R., et al., 2022. Interspecies and seasonal variations in macroalgae from the Nordic region: chemical composition and impacts on rumen fermentation and microbiome assembly. *J. Clean. Prod.*, 132456.
- Paul, A., Frederich, M., Megido, R.C., et al., 2017. Insect fatty acids: a comparison of lipids from three Orthoptera and *Tenebrio molitor* L. larvae. *J. Asia Pac. Entomol.* 20 (2), 337–340.
- Pinotti, L., Luciano, A., Ottoboni, M., et al., 2021. Recycling food leftovers in feed as opportunity to increase the sustainability of livestock production. *J. Clean. Prod.* 294, 126290.
- Przemieniecki, S.W., Kosewska, A., Ciesielski, S., et al., 2020. Changes in the gut microbiome and enzymatic profile of *Tenebrio molitor* larvae biodegrading cellulose, polyethylene and polystyrene waste. *Environ. Pollut.* 256, 113265.
- Quast, C., Pruesse, E., Yilmaz, P., et al., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41 (D1), D590–D596.
- Rajagopal, R., 2009. Beneficial interactions between insects and gut bacteria. *Indian J. Microbiol.* 49 (2), 114–119.

- Raney, T., Skoet, J., Steinfeld, H., 2009. The State of Food and Agriculture: Livestock in the Balance. Food and Agriculture Organization of the United Nations, Rome, Italy.
- Rao, P.R., Metta, V.C., Johnson, B.C., 1959. The amino acid composition and the nutritive value of proteins: I. Essential amino acid requirements of the growing rat. *J. Nutr.* 69 (4), 387–391.
- Rapin, A., Pattaroni, C., Marsland, B.J., et al., 2017. Microbiota analysis using an Illumina MiSeq platform to sequence 16S rRNA genes. *Curr. Protoc. Mol. Biol.* 7 (2), 100–129.
- Ravzanaadii, N., Kim, S.-H., Choi, W.-H., et al., 2012. Nutritional value of mealworm, *Tenebrio molitor* as food source. *International Journal of Industrial Entomology* 25 (1), 93–98.
- Razdan, A., Pettersson, D., 1994. Effect of chitin and chitosan on nutrient digestibility and plasma lipid concentrations in broiler chickens. *Br. J. Nutr.* 72 (2), 277–288.
- Robertson, J., 1981. The detergent system of analysis and its applications to human foods. The analysis of dietary fiber in food 123–158.
- Rui, L., 2013. *Brevibacillus laterosporus*, a pathogen of invertebrates and a broad-spectrum antimicrobial species. *Insects* 4 (3), 476–492.
- Rumbos, C.I., Karapanagiotidis, I.T., Mente, E., et al., 2020. Evaluation of various commodities for the development of the yellow mealworm, *Tenebrio molitor*. *Sci. Rep.* 10 (1), 1–10.
- Rumpold, B.A., Schlüter, O.K., 2013. Nutritional composition and safety aspects of edible insects. *Mol. Nutr. Food Res.* 57 (5), 802–823.
- Ruschioni, S., Loreto, N., Foligni, R., et al., 2020. Addition of olive pomace to feeding substrate affects growth performance and nutritional value of mealworm (*Tenebrio molitor* L.) larvae. *Foods* 9 (3), 317.
- Sánchez-Muros, M.-J., Barroso, F.G., Manzano-Agugliaro, F., 2014. Insect meal as renewable source of food for animal feeding: a review. *J. Clean. Prod.* 65, 16–27.
- Shin, C.-S., Kim, D.-Y., Shin, W.-S., 2019. Characterization of chitosan extracted from Mealworm Beetle (*Tenebrio molitor*, Zophobas morio) and Rhinoceros Beetle (*Allomyrina dichotoma*) and their antibacterial activities. *Int. J. Biol. Macromol.* 125, 72–77.
- Smith, D.M., 2017. Protein Separation and Characterization Procedures. Food Analysis. Springer, pp. 431–453.
- Song, Y.-S., Kim, M.-W., Moon, C., et al., 2018. Extraction of chitin and chitosan from larval exuvium and whole body of edible mealworm, *Tenebrio molitor*. *Entomol. Res.* 48 (3), 227–233.
- Tabata, E., Kashimura, A., Kikuchi, A., et al., 2018. Chitin digestibility is dependent on feeding behaviors, which determine acidic chitinase mRNA levels in mammalian and poultry stomachs. *Sci. Rep.* 8 (1), 1–11.
- Team, R.C., 2021. R: A Language and Environment for Statistical Computing.
- Thrastardottir, R., Olafsdottir, H.T., Thorarinsdottir, R.I., 2021. Yellow mealworm and black soldier fly larvae for feed and food production in Europe, with emphasis on Iceland. *Foods* 10 (11), 2744.
- Tilley, J., Terry, D.R., 1963. A two-stage technique for the in vitro digestion of forage crops. *Grass Forage Sci.* 18 (2), 104–111.
- Toviho, O.A., Bársony, P., 2022. Nutrient composition and growth of yellow mealworm (*Tenebrio molitor*) at different ages and stages of the life cycle. *Agriculture* 12 (11), 1924.
- Urbanek, A.K., Rybak, J., Wróbel, M., et al., 2020. A comprehensive assessment of microbiome diversity in *Tenebrio molitor* fed with polystyrene waste. *Environ. Pollut.* 262, 114281.
- Van Broekhoven, S., Oonincx, D.G., Van Huis, A., et al., 2015. Growth performance and feed conversion efficiency of three edible mealworm species (Coleoptera: Tenebrionidae) on diets composed of organic by-products. *J. Insect Physiol.* 73, 1–10.
- Veldkamp, T., Bosch, G., 2015. Insects: a protein-rich feed ingredient in pig and poultry diets. *Animal Frontiers* 5 (2), 45–50.
- Wigglesworth, V., 1988. The source of lipids and polyphenols for the insect cuticle: the role of fat body, oenocytes and oenocytoids. *Tissue Cell* 20 (6), 919–932.
- Wynants, E., Crauwels, S., Lievens, B., et al., 2017. Effect of post-harvest starvation and rinsing on the microbial numbers and the bacterial community composition of mealworm larvae (*Tenebrio molitor*). *Innovat. Food Sci. Emerg. Technol.* 42, 8–15.
- Wysokowski, M., Petrenko, I., Stelling, A.L., et al., 2015. Poriferan chitin as a versatile template for extreme biomimetics. *Polymers* 7 (2), 235–265.
- Yoo, J., Cho, K., Hong, J., et al., 2019. Nutrient ileal digestibility evaluation of dried mealworm (*Tenebrio molitor*) larvae compared to three animal protein by-products in growing pigs. *Asian-Australas. J. Anim. Sci.* 32 (3), 387.
- Yu, X., Oh, E., Kim, Y., 2021. A comparative study on the functional properties of mealworm (*Tenebrio molitor*) larvae and soybean protein isolates and hydrolysates. *International Food Research Journal* 28 (4).
- Zhang, Q., Hou, Y., Bazer, F.W., et al., 2021. Amino Acids in Swine Nutrition and Production. *Amino Acids in Nutrition and Health*. Springer, pp. 81–107.
- Zhang, X., Tang, H., Chen, G., et al., 2019. Growth performance and nutritional profile of mealworms reared on corn stover, soybean meal, and distillers' grains. *Eur. Food Res. Technol.* 245 (12), 2631–2640.
- Zhao, X., Vazquez-Gutierrez, J.L., Johansson, D.P., et al., 2016. Yellow mealworm protein for food purposes-extraction and functional properties. *PLoS One* 11 (2).
- Zielińska, E., Baraniak, B., Karaś, M., et al., 2015. Selected species of edible insects as a source of nutrient composition. *Food Res. Int.* 77, 460–466.
- Zielińska, E., Zieliński, D., Jakubczyk, A., et al., 2021. The impact of polystyrene consumption by edible insects *Tenebrio molitor* and *Zophobas morio* on their nutritional value, cytotoxicity, and oxidative stress parameters. *Food Chem.* 345, 128846.