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Polyethylene, polystyrene and lignocellulose wastes as mealworm (*Tenebrio molitor* L.) diets and their impact on the breeding condition, biometric parameters, metabolism, and digestive microbiome

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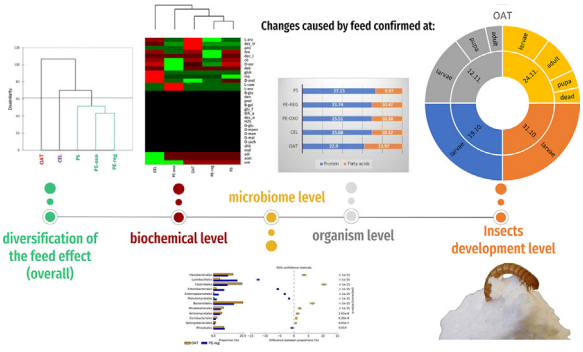
HIGHLIGHTS

- The mealworm larvae can successfully consume polystyrene, polyethylene and cellulose.
- Best diet for mealworm larvae growth is polystyrene, then polyethylene, worst is cellulose.
- Of the tested diets regranulated polyethylene is the best substrate for mealworm larvae metamorphosis.
- Each diet affects the gut microbiome and biochemistry differently.

GRAPHICAL ABSTRACT



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ABSTRACT

This study aimed to identify the extent to which a diet of oatmeal and polymers affects the development of mealworms, their microbiome, the biochemical activity of their digestive system, and their feed-metabolizing capacity. With a polystyrene diet, feed loss was most significant, as indicated by FTIR (Fourier-transform infrared spectroscopy) of frass, which showed that polystyrene was the only compound that was chemically modified. Compared to the control diet, mealworm larvae developed best on polyethylene regranulate (PE-reg), quickly transiting from one developmental stage to another with minor mass loss. A lignocellulose-based diet was the least beneficial for mealworm development. A polystyrene diet was most beneficial in terms of the protein content in larvae, but the contents and quality (usefulness as food) of fatty acids in the insects fed these wastes were significantly lower than in the control insects. For each diet, specific microbial cultures formed, and the presence of protozoa and various biochemical activities suggested different survival strategies and assimilation mechanisms facilitating survival. Despite profound changes in the microbiota and biochemistry of the digestive tract of mealworms fed waste-based diets, this study indicates their potential for utilizing PE-reg and polystyrene.

1. Introduction

A drastic increase in the human population, industry and consumptive lifestyles is increasingly contributing to environmental problems (Moore et al., 2011). Humans produce more and more products that are not fully consumed and quickly turn into waste (Renou et al., 2008). In recent

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decades, plastics have become particularly prized materials with a wide range of uses. They are used, inter alia, in the manufacturing of packaging material, in construction and aviation, as well as in the production of machines and vehicle components. Plastics owe their popularity to features such as plasticity, strength, and durability while also being light and cost-effective (Thompson et al., 2009; Kalwa et al., 2019; Paxton et al., 2019).

In 2019, the global production of plastic was estimated at approx. 370 million tonnes. This is almost six times more than the 1989 total of 64 million tonnes. Today, Europe alone produces this much, making up approx. 16% (60 million tons) of the world production (Pielichowski and Puszyński, 1998; Plastics Europe, 2020).

As a testament to the output, the total amount of plastics released by humans into the ecosystem is more than 8300 million tons (Geyer et al., 2017). Additionally, the demand for plastics is constantly growing. The amount of plastics in circulation is projected to increase to 417 million tones per year by 2030 (Hundertmark et al., 2018). A particularly problematic type of plastic is polystyrene. About 35 million tons of polystyrene are produced annually, an amount that continues to increase by about 5% per year on average (Baker, 2018).

By employing linear material flows, i.e., extracting raw materials, making products, using them, and throwing them away, humans contribute to the continuous depletion of natural resources and increased production of waste (Nowak and Cybulska, 2019). Plastic waste is not the only problem for humanity — all types of waste, including those based on lignocellulose, need to be managed. In nature, compounds undergo cyclical changes, and methods are constantly being sought to harness and restore normal circulation of matter (Kostecka et al., 2014).

Increasing awareness of the use of plastics and the correct segregation and disposal thereof is undoubtedly an essential step towards reaching sustainable development goals (Juda-Rezler and Manczarski, 2010; Malizia and Monmany-Garzia, 2019). One of the ways to manage plastic waste is to process it using insects, which by eating and digesting these materials, accelerate the decomposition process. Examples of insects that have been shown to digest plastic are the mole caterpillar (*Galleria mellonella*), the breadfruit (*Plodia interpunctella*), and some species from the Tenebrionidae family such as mealworms (*Tenebrio molitor*), superworms (*Zophobas morio*), *Tenebrio obscurus*, *Tribolium castaneum*, or *Alphitobius diaperinus* (Bombelli et al., 2017; Ritter, 2017; Peng et al., 2019; Wang et al., 2020; Cucini et al., 2020; Yang et al., 2021).

As a food source, insects have also undergone research with respect to food safety. Poma et al. (2017) showed that insect contamination with undesirable organic substances such as PCBs, OCPs, BFRs, PFRs and dioxin, as well as As, Cd, Co, Cr, Cu, Ni, Pb, Sn, and Zn is comparable to or lower than in that in meat products or fish. Pesticide residues have been identified in a relatively large number of insects, but the overall characteristics of insect biomass compare well with that of other food products.

Particular attention has been given to mealworm larvae, which are distinguished by their voracity, resistance to unfavorable environmental conditions, and rapid biomass growth (Siemianowska et al., 2013). Nevertheless, it should be remembered that the mealworm is an acceptable food source for humans and animals (Gałęcki et al., 2021); therefore, the impact of contamination of insect feed with plastic may indirectly affect other organisms, including humans. The features mentioned above have given the mealworm the ability to digest foods that are not naturally present in the environment, including polyethylene, polystyrene, and processed cellulose, as confirmed by many studies (Nukmal et al., 2018; Yang et al., 2018b; Vigneron et al., 2019). Even though plastics are durable and resistant to damage and biodegradation, these insects can use them as food. Mealworms can chew plastic thanks to their strong mandibles (Hassan et al., 2016). Their gut contains symbiotic microbiota that support the digestive process, playing a crucial role in the digestion of difficult-to-degrade plastics (Yang et al., 2015; Przemieniecki et al., 2020; Urbanek et al., 2020). Thanks to this microbiota (and a with the addition of cereal grains), the larvae can adapt to digesting various foods including synthetic polymers (Wang and Zhang, 2015; Brandon et al., 2018; Peng et al., 2021). In addition to synthetic polymers, one of the foods that the mealworm can digest is processed lignocellulose (e.g., cardboard), which according to Willis et al. (2010), is an ability noted in few insect species. In their studies, He et al. (2021) also revealed new insight into lignocellulosic crop waste utilization that involves insects. This process is similar to the digestion of polystyrene. The intestinal microbiota of mealworm larvae can degrade celluloses, which are found in resistant cellulose waste (Huang et al., 2012). Nevertheless, the inclusion of mealworms and other insects in the food chain of humans and livestock has necessitated the development of new, rapid methods for detecting dangerous insect products such as plastics. The best-known methods are FTIR (mentioned above) and the gas-chromatography–mass-spectrometry Untargeted Screening Method. These methods not only detect polystyrene, but also indicate its degree of degradation (Brandon et al., 2018; Tsochatzis et al., 2021a).

This study aimed to identify disturbances in the functioning of mealworm (*Tenebrio molitor* L.) cultures by determining how a diet of polyethylene, polystyrene, and lignocellulose affects breeding and changes the metabolism and microbiome structure of the larval digestive system. Additionally, it assessed the mealworms' potential to process such wastes concerning insects welfare.

2. Materials and methods

2.1. Sampling design

The study was conducted using mealworm larvae from the collection of the Department of Entomology, Phytopathology and Molecular Diagnostics of the University of Warmia and Mazury in Poland. Each experimental group was made up of 100 larvae with a body length of about 1.5 cm. The larvae originated from one breeding and developed under the same conditions. It was assumed that in addition to their physical condition, their body length would also serve as an indicator of similar stages of development (between the 7th and 10th instars).

Larvae were placed in glass jars with previously prepared substrates, consisting of cardboard (CEL), polystyrene (PS), oxo-degradable polyethylene (PE-oxo), polyethylene regranulate (PE-reg), or oatmeal (OAT) as a control feed. In the experiments, the insects were fed mono-diets; therefore, substrates were provided individually. The nutritional values per 100 g of oatmeal (flakes with bran) were: protein, 12 g; fat, 7.2 g (saturated fatty acids, 1.3 g); carbohydrates, 62 g including 12.9 g of fiber; and mineral substances, 5.9 g (data obtained from oatmeal producer). This diet was selected because it was the basic diet of the insect breeding line prior to the start of the experiment. Moreover, the breeding indicators were satisfactory, and the use of a different reference feed could have disturbed the structure and functioning of the core microbiota developed in the digestive tract. The cardboard diet consisted of shredded packaging cardboard with a density ~ 0.36 g cm^3. Both polyethylene diets (PE-oxo, PE-reg) consisted of polyethylene with a density ~ 0.92 g cm^3, additionally PE-oxo contained ~1% starch as additive. The partially recovered polyethylene (polyethylene regranulate – PE-reg) contained ~2% of CaO and $CaCO_3$-based pigments. The polystyrene diet (PS) consisted of shredded white styrofoam, used for packaging, with a density ~ 0.02 g cm^3. The jars in which the insects were bred were secured with milling gauze to prevent the escape of insects and to maintain adequate air circulation. The ambient temperature was 25°C, and the humidity ranged from 60 to 72%. The experiment was carried out in triplicate with each substrate (5 substrate variants × 3 replications × 100 larvae). This study on growing mealworm larvae on various substrates was carried out over 45 days (the 2-month mortality test was performed separately). After 45 days of the experiment, the live larvae were collected for further analysis (protein content, fatty acids, genetic and biochemical digestive tract analysis). Biometric measurements were also made: live larvae mass, single individual mass, and number of adults, pupae and larvae. The waste substrate and frass were weighed to determine the effectiveness of waste degradation.

2.2. Protein content

From five to six larvae with total mass of one gram were sampled from each diet group and frozen at −80 °C and then homogenized in a mortar. For the next step, 100 mg of the resultant powder was mixed with 20 ml of demineralized H_2O. Acid hydrolysis was then performed. The lysates were stirred at 300 rpm for 30 min, then centrifuged at 4 °C and 8000 $\times g$ for 20 min. The determination of protein content was performed using the Bradford method (Bradford, 1976). For this, 800 μl of the obtained supernatant (protein extract) was mixed with 200 μl of Bradford's reagent (Sigma-Aldrich, Germany) and incubated at ambient temperature for 20 min. Absorbance was measured using a NanoDrop 2000C spectrophotometer at 595 nm. The blank was prepared using demineralized H_2O, and the standard curve was generated from a known concentration of bovine serum albumin (Sigma-Aldrich, Germany). Protein content calculations were made using an on-line calculator (Quest Graph™ Bradford Protein Assay Calculator https://www.aatbio.com/tools/bradford-protein-assay-calculator).

2.3. Fatty acid content and profile

Crude fat was extracted using a Soxhlet extractor (according to standard PN-ISO 6492: 2005). A portion (1 g) of the air-dried and homogenized whole insects was extracted with petroleum ether in a Soxhlet extractor for 6–8 h until the fat was entirely removed. The ether was then distilled off from the extract (in the same apparatus), and the crude fat residue (ether extract) was dried in an oven and weighed.

Fatty-acid methyl esters were prepared to determine fatty acid composition according to the modified Peisker method (methanol: chloroform: concentrated sulfuric acid at a ratio of 100:100:1, v/v) (Żegarska et al., 1991). Separation and determination of fatty acids was carried out by gas chromatography using a Varian CP-3800 gas chromatograph with a flame ionization detector (FID), a 50 m long capillary column (internal diameter: 0.25 mm, film thickness: 0.25 μm) and a dispenser (50:1 split). The sample size was 1 μl. The operation conditions were as follows: detector temperature 250 °C, injector temperature 225 °C, column temperature 200 °C, carrier gas: helium (1.2 ml min^{-1} flow). Individual standards of fatty acid methyl esters (Sigma-Aldrich) were used in the study. The relative content of fatty acids was expressed as % of the total area of all fatty acid peaks contained in the sample.

2.4. DNA isolation

After the completion of the biometric measurements, the digestive tract was prepared (~100 mg individually from each jar). The isolation method described by Hermann-Bank et al. (2013) and Tkacz et al. (2018) was used to extract DNA for the study. Larval digestive tracts were excised (100 mg) after two months of feeding. DNA was isolated from the collected material with a QIAamp PowerFecal DNA Kit (Qiagen, Germany) and concentrations for each sample were quantified by fluorometric quantitation using a Quantus™ Fluorometer (Promega, Germany).

2.5. Quantitative PCR

Information relating to Real-Time q-PCR is presented in Table A1 and Table A2 based on previous work (Przemieniecki et al., 2021).

2.6. 16S rRNA amplicon sequencing and bioinformatics analysis

The microbial communities in the analyzed samples were examined by sequencing of the V3-V4 region of the 16S rRNA gene. The 16S rRNA gene fragment was amplified with the PCR primers recommended for the Illumina method. The primers were designed by adding Illumina adapter overhang nucleotide sequences to the PCR primers provided by Klindworth et al. (2013). Amplicons were indexed using a Nextera® XT Index Kit according to the manufacturer's instructions. DNA was sequenced in Illumina MiSeq in 2 × 250 paired-end mode. The results of the sequencing were saved in FASTQ files and uploaded to the MetaGenome Rapid Annotation Subsystems Technology (MG-RAST) server for analysis (Meyer et al., 2008). Each file underwent quality control (QC) which included quality filtering (removing sequences with ≥5 ambiguous base pairs) and length filtering (removing sequences with a length ≥ 2 standard deviations from the mean). The Illumina metagenomic datasets are available at MG-RAST under accession numbers 4785882.3 (OAT), 4785878.3 (CEL), 4785889.3 (PE-oxo), 4785887.3 (PE-reg), 4785884.3 (PS).

2.7. Biochemical properties of larvae gut

The biochemical activity and carbon source take-up in larval digestive tracts were determined using API 20 E and API 20 NE tests, respectively. Samples of ~100 mg were collected from each variant and homogenized in vials containing glass beads and 1 ml of peptone water. The samples were homogenized in a TissueLyser LT bead homogenizer (Qiagen, Germany) at 30 oscillations for 5 min. The resulting suspension was diluted 1:10 in peptone water with 1% TSB (Triptic soy broth. Merck, Germany), incubated at 28 °C for 4 h and used in API tests, in accordance with the manufacturer's instructions. Biochemical tests were incubated at 28 °C for 24 h, and metabolic tests were incubated at 28 °C for 18 h. The colorimetric and optical density results were interpreted based on standards and methodology provided by the producer (Biomerieux, France).

The chemical changes of synthetic polymers during digestion were identified using FTIR (Fourier-transform infrared spectroscopy). For detection of IR absorption, raw samples of frass were placed in a FTIR detector (Thermo Scientific™ Nicolet™ iS20 FTIR Spectrometer). The plastics fed to the mealworms were used as controls for the respective frass samples. The results were collected and analyzed using OMNIC v9 software.

2.8. Statistical analysis

The results were first tested for normality of the data distributions (Shapiro-Wilk method). Differences between the means of biometric parameters were assessed with one-way analysis of variance (ANOVA). Differences between individual groups were examined with Duncan's test ($p < 0.05$). Groups without statistically significant differences were assigned an identical letter, e.g., a, b, c, etc. Groups of means assigned with different letters were significantly different from each other.

The correlations between the experimental variants and the studied variables were examined using principal components analysis (PCA). The dissimilarity between variants was analyzed with agglomerative hierarchical clustering (AHC) on the basis of a dissimilarity matrix calculated with the Bray-Curtis method.

The results were processed statistically and presented graphically in XLSTAT (Addinsoft), Statistica v. 13.3 (StatSoft, 2013) and Canoco v. 4.5. Taxonomic differences between metagenomes were analyzed using Statistical Analysis of Metagenomic Profiles (STAMP v. 2.1.3) (Parks and Beiko, 2010). Statistically significant differences between metagenomes were identified by Fisher's exact test combined with the Newcombe-Wilson method for calculating confidence intervals.

3. Results

Table 1 shows the biometric results for *T. molitor* after 45 days of growth according to feed type. Statistically significant differences were observed between larval counts across the tested variants ($F = 8.92$; $p = 0.002$). The lignocellulose (80.09%) and PE-oxo (79.83%) diets had the highest larval counts, and the PS variant (77.62%) also had a high count. The oat flakes (46.11%) and PE-reg (59.6%) diets had by far the lowest counts.

Analysis of the pupa numbers also showed statistically significant differences between the studied diets ($F = 8.19$; $p = 0.003$). The OAT variant (17.3%) had the highest proportion of live individuals, the CEL and PE-oxo variants had the lowest proportions (approx. 5%).

Table 1
Biometrical parameters of *Tenebrio molitor* stages.

Parameter*	OAT	CEL	PE-oxo	PE-reg	PS	F	*p*
Proportion of live individuals after 45 days of the experiment (%)							
Larvae	46.11 ± 6.66 b**	80.09 ± 3.73 a	79.83 ± 4.79 a	59.60 ± 6.15 b	77.62 ± 3.26 a	8.92	0.002
Pupa	17.29 ± 1.01 c	4.81 ± 1.86 a	5.55 ± 1.29 a	12.57 ± 2.89 bc	9.55 ± 1.33 ab	8.19	0.003
Imago	36.61 ± 5.69 b	15.10 ± 3.79 a	14.62 ± 3.54 a	27.83 ± 6.06 ab	12.83 ± 2.99 a	5.14	0.016
Mass of live larvae (g)							
Total	9.36 ± 0.3 c	7.70 ± 0.28 a	7.37 ± 0.32 a	9.02 ± 0.09 bc	8.25 ± 0.44 ab	7.53	0.005
One larvae***	0.24 ± 0.04 c	0.11 ± 0.008 a	0.12 ± 0.018 ab	0.22 ± 0.05 bc	0.14 ± 0.02 ab	4.11	0.031
Change in the mean mass of one live larva during the experiment (%)****							
	43	−15	0	32	14		
Total biomass of live larvae at the end of experiment (g)							
	28.07	23.09	22.10	27.06	24.74		
Mean number of dead individuals during the experiment							
	13.0 ± 2.0	9.33 ± 0.88	25.33 ± 3.67	25.67 ± 9.84	27.68 ± 4.845		

* Abbreviations: OAT – oatmeal, CEL – cardboard, PE-oxo – oxo-degradable polyethylene, PE-reg – polyethylene regranulate, PS – polystyrene; ML – Mass of larvae; ML 1_B – Mass of one larva at the beginning of the experiment; ML 1_E – Mass of one larva at the end of the experiment.
** Mean ± SE;
*** Mass of each larvae at the beginning of experiment was 12.5 ± 0.5 g (not significant).
**** Percentage data in relation to 100% i.e. the mass of live larvae present at the start of the experiment.

The results of the pupal count analysis also differed significantly between the studied diets ($F = 8.19$; $p = 0.003$). The OAT variant had the highest share (17.3%), and the CEL and PE-oxo variants had the lowest (approx. 5%).

The share of adults was also significantly different across variants ($F = 5.14$; $p = 0.01$), with the highest shares of imagines in the OAT (36.6%) and PE-reg (27.8%) variants, the lowest in the PS (12.8%) variant, and slightly higher shares in the PE-oxo and CEL variants (approx. 15%).

The mass of the larvae also differed significantly between the tested variants ($F = 7.53$; $p = 0.004$). The OAT (9.36 g) and PE-reg (9.02 g) groups had the highest larval mass, the PE-oxo had the lowest (7.37 g). Although the mean mass of individual (single) larvae did not differ significantly at the beginning of the experiment, their mean mass differed significantly at the end ($F = 4.11$, $p = 0.031$). The oatmeal (0.24 g) and PE-reg (0.22 g) diets had the highest average mass of individual larvae; the lignocellulose diet had the lowest (0.11 g). The mean mass of individual larvae fed with oatmeal increased by 43% from the beginning to the end of the experiment. For comparison, the mean mass of those fed with PE-reg increased 32%, whereas the mean mass of larvae fed lignocellulose decreased 15%.

The Agglomerative Hierarchical Clustering results regarding the development of the insects indicated that the OAT and PE-reg groups were similar, as were the CEL and PE-oxo groups, but the PS group differed from all other groups (Fig. 1). Nevertheless, the oatmeal-fed larvae had the lowest counts at the last measurement. However, a low number of larvae was associated with a higher metamorphosis rate, and the highest number of pupae and adults emerging during the experiment. The oatmeal (OAT) diet also produced fewer dead insects during the experiment. Nevertheless, the lowest number of dead insects and the highest number of larvae, combined with simultaneous severe loss of body mass at last measurement, were observed in the lignocellulose group. The insects growing on the PE-reg diet had a development pattern similar to that exhibited in the OAT diet group, expressed as the potential for faster transformation to the next developmental stage. However, it should be noted that the PS and PE-oxo variants were characterized by high insect mortality.

These results are supplemented with the analysis of breeding growth dynamics over time (Fig. B). The fastest metamorphosis and moderate mortality characterized OAT based breeding. After 45 days of breeding, the number of adults was the highest in the OAT and CEL variants. Nevertheless, the rate of consumption and activity of insects in the lignocellulose diet were low, which could suggest that rapid metamorphosis was a means of escape from an adverse environment. In the PE-oxo and PS variants, metamorphosis was the lowest. Mortality was highest in PS and PE-reg, and slightly lower in PE-oxo. Nevertheless, the PE-reg group produced a relatively high number of adults and satisfactory biometric parameters, which may suggest increased cannibalism in this population. This is largely confirmed by the proportion of the pupae, which are easy prey. In the PE-reg variant, the number of pupae was highest on day 45 (over 36% of specimens). Unfortunately, the number of adults at the end of the experiment was much smaller than expected.

Protein content in larvae fed was highest by far in the PS group (27.2%) and lowest for in the OAT group (22.9%). In the other variants, it averaged 23.6% and was significantly lower in the PS group (Table 2). FA content exhibited the opposite trend to that of protein. The results were significantly different between the highest- and lowest-value groups, OAT (14%), and PS (10%), respectively. No significant differences were noted between the other variants, with the mean FA content being 10.4% (Table 2).

The fatty acid profile analysis (Table 3, Fig. 1D) showed that the FA composition was homogenous within the PE and CEL groups. Oleic acid (C18:1 c9) accounted for about 50% of the fatty acid content. Nevertheless, a certain discrepancy was observed between the FA profile of the OAT-group larvae and those of larvae fed other diets. The most important difference was the change in the content of linoleic acid, which was about 20% in the OAT larvae and about 27% in the other variants. On the other hand, the opposite relationship was observed for palmitic acid, whose percentage in the OAT diet was 16.8%, and about 12% in the other variants. Similarly, higher shares of FA were found in the OAT variant than the other variants: stearic acid, 3.7 vs. 2.8%; palmitoleic acid, 2% vs 1.6%; marganic acid, 0.22 vs, 0.14%; and arachidic acid, 0.2 vs. 0.1%. Additionally, the fraction of myristoleic acid was found to be smaller (4.8%) in the PS variant than in the others (around 5.6%). The share of α-linoleic acid in PS (almost 0.5%) was higher than in the OAT-fed larvae (slightly over 0.1%). There was also an apparent reduction of margaroleic acid in the PE-reg variant, which amounted to less than 0.1%, whereas in the other variants it was close to 0.3% (Table 3).

The analysis of fatty acid saturation showed that the experimental variants only differed in terms of SFAs (saturated fatty acids) and PUFAs (polyunsaturated fatty acids). SFAs were found at higher levels (27.4%) in the OAT-fed larvae than in the other larvae (about 21%), with the lowest value observed in the PS variant (20.3%). PUFAs were lowest in OAT (20.3%) and highest in PS (27.7%), with values of about 27% in the remaining variants. The concentration of MUFAs was similar across all variants and averaged 52.1%; only the larvae fed with PE-reg displayed a slightly lower percentage of this FA group (51.8%). Nevertheless, both the FA profile analysis data and the global analysis (AHC) indicated that OAT is a completely separate variant regarding the quantity and quality of fatty acids than CEL and both PE. Furthermore, these analyses also showed

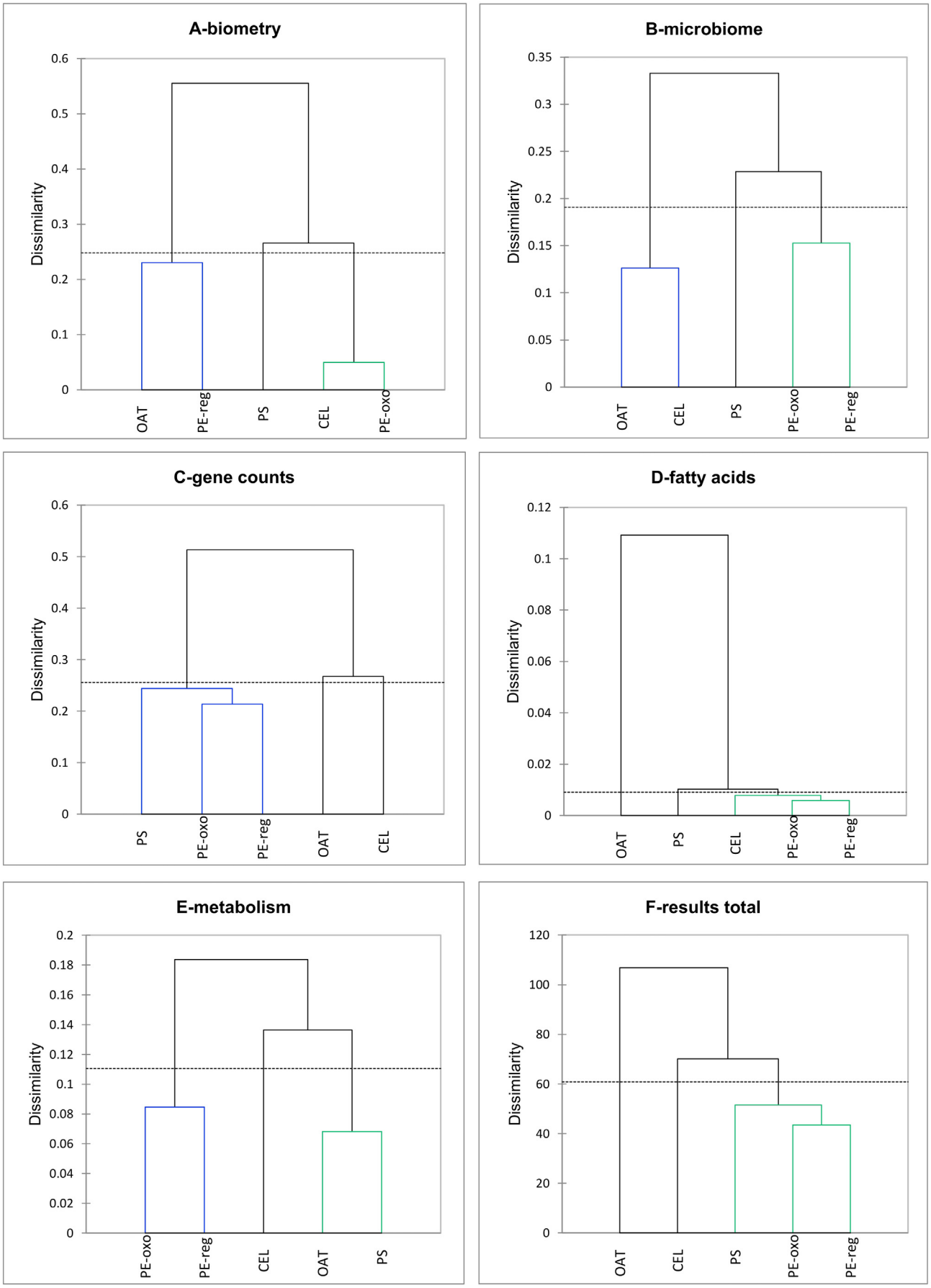

Fig. 1. Agglomerative Hierarchical Clustering of dissimilarity between *Tenebrio molitor* diets based on the individual results of the analyzes performed after the experiment (Diets descriptions: OAT – oatmeal, CEL – cardboard, PE-oxo – oxo-degradable polyethylene, PE-reg – polyethylene regranulate, PS – polystyrene).

Table 2
The content of total protein and crude fat in live larvae determined after experiment, by diet (OAT – oatmeal, CEL – cardboard, PE-oxo – oxo-degradable polyethylene, PE-reg – polyethylene regranulate, PS – polystyrene).

Diet	Protein (%)	Fatty acid (%)
OAT	22.90b	13.97a
CEL	23.68b	10.32ab
PE-oxo	23.51b	10.38ab
PE-reg	23.74b	10.42ab
PS	27.15a	9.97b

that the PE variants form a relatively homogeneous group, while PS is a separate variant, more similar to a diet based on polyethylene and lignocellulose than one based on OAT (Table 3, Fig. 1D).

A preliminary difference analysis (clustering) of the results for the diets (Fig. 1) shows that they can be divided into 3 groups based on biometry, i.e., OAT + PE-reg, PS, and CEL + PE-oxo. Microbiome-focused clustering also produced 3 groups: OAT + CEL, PS, and PE-oxo + PE-reg. The clustering of qPCR results (number of gene copies) coincided relatively well with the Euclidean clustering of all results, creating a PS + PE-oxo + PE-reg group and two others: OAT and CEL. Grouping diets in terms of their fatty acid profile resulted in the creation of 3 groups: PS and the related CEL + PE-oxo + PEreg, and OAT (clearly different from the others). Three groups were distinguished in terms of metabolism: PE-oxo + PE-reg, CEL, and OAT + PS. The Bray-Curtis dissimilarities were greatest (from approx. 0.34 to 0.56) for biometrics, number of genes and the microbiome, while the dissimilarity of metabolism and fatty acids was below 1.2. The dissimilarity of the overall results, based on the grouping of Euclidean distances, was high (approx. 100) – a finding similar to that obtained in the previous study (Przemieniecki et al., 2020).

Based on the results of the variability analysis of selected biochemical parameters (Fig. 2), it was found that 15 out of 31 traits showed similar activity, and these were determined to be the core traits (constants not affected by the treatment). In the case of differentiation of traits (left dendrogram), there were 7 branches, indicating dissimilarity of traits depending on the diet and activity levels. As for the similarity of diet (upper dendrogram), the similarity decreased from right to left (the least similar between PS and CEL, and the most between PS and PE-reg). The OAT variant (no or low activity of L-arabinose, tryptophan deaminase, amygdalin, phenylacetic acid, trisodium citrate and urease) was characterized by low activity, but it strongly utilized D-melibiose. The distinguishing feature of the PS variant was the capacity to utilize inositol and the low activity of D-sorbitol, phenylacetic acid, trisodium citrate and urease. The larvae in the PE-reg variant showed high utilization of L-arabinose, phenylacetic acid, D-sorbitol and tryptophan deaminase. However, they showed no significant urease activity. The PE-oxo variant was characterized by high utilization of trisodium citrate, D-sorbitol and decanoic acid, as well as tryptophan deaminase and urease. In this variant, the microbiome was distinguished from the other variants by a lack of L-rhamnose and L-arabinose utilization. The CEL variant was the greatest outlier in terms of larval digestive system capacity, showing high acetoin production potential, urease activity and utilization of adipic acid. It also showed no or low capacity to utilize L-arabinose, phenylacetic acid, inositol, trisodium citrate, potassium gluconate and D-melibiose (Fig. 2). L-arabinose is an intermediate of the pentose phosphate pathway, which flows through the pathways of central metabolism to satisfy the cell's need for precursor metabolites, reducing power, and metabolic energy; this is a standard cell strategy during intensive digestion.

Table 3
Fatty acid fractions in total lipid content (%) in *Tenebrio molitor* live larvae determined after experiment, fed with different foods (OAT – oatmeal, CEL – cardboard, PE-oxo – oxo-degradable polyethylene, PE-reg – polyethylene regranulate, PS – polystyrene).

Fatty acid		OAT	CEL	PE-oxo	PE-reg	PS
C12:0	Lauric acid	0.60	0.32	0.29	0.31	0.24
C14:0	Myristoleic acid	5.71	5.69	5.29	5.31	4.83
C15:0	Pentadecanoic acid	0.03	0.08	0.08	0.08	0.08
C16:0	Palmitic acid	16.84	11.92	11.91	12.15	12.01
C16:1	Palmitoleic acid	2.03	1.68	1.60	1.61	1.67
C17:0	Margaric acid	0.22	0.14	0.13	0.15	0.15
C17:1	Margaroleic acid	0.27	0.26	0.25	0.07	0.25
C18:0	Stearic acid	3.69	2.69	2.69	2.92	2.81
C18:1 *c9*	Oleic acid	49.95	50.2	50.36	50.01	50.07
C18:2	Linoleic acid	20.2	26.43	26.85	26.79	27.23
C18:3	α-Linoleic acid	0.13	0.37	0.32	0.33	0.43
C20:0	Arachidic acid	0.18	0.09	0.10	0.10	0.11
C20:1	Gondoic acid	0.06	0.07	0.07	0.07	0.07
C22:0	Behenic acid	0.08	0.06	0.06	0.09	0.06
	SFA	27.36	20.99	20.55	21.11	20.29
	MUFA	52.31	52.21	52.28	51.77	52.06
	PUFA	20.33	26.80	27.17	27.13	27.65

The structure of the bacterial microbiome at the row level was moderately varied (Fig. 1B, Fig. 3; dissimilarity at 0.34). Generally speaking, the orders Bacteroidales (mean 17.4%) and Actinomycetales (mean 5.3%) were the most common. These orders exhibited higher abundance in OAT (20.9 and 6.0%, respectively) and CEL (19.9 and 6.1%, respectively). There were also high fractions of Clostridiales (14.5% on average), which were higher in the OAT diet, and significantly lower in the PE-oxo diet compared with all other variants. The greatest fraction of Rhizobiales (mean 8.6%) was found in the PE-reg group. Lactobacillales, with an average of 10.5%, were at least twice as prolific in the PE-oxo, PEreg and PS groups (mean 13.4%) than in OAT and CEL (mean 6.2%). Selenomonadales levels (mean 3.0%) were the highest in the CEL group, whereas Rhodobacterales levels (mean 3.9%) were clearly the highest in the OAT (7.2%) and PE-oxo (5.2%) groups. The orders of Nitrospirales (average 1.5%), Nitrosomonadales (1.6%) and Planctomycetales (9.9%) had the highest levels by far (about fourfold) in the PS variant. The reverse was observed for Coriobacteriales (1.8% on average), Burkholderiales (2.0%), Enterobacteriales (4.5%), with fractions clearly lower in the PS group, though Enterobacteriales were the only order highly abundant in the PE-based groups (about 8%). The order Pseudomonadales generally formed a small part of the microbial communities, with an average fraction of 1.4%, which was still 4 times higher in the PE-reg and PS variants than the others. The abundance of Entomoplasmatales was 1.1% on average, but significantly higher in the PE-oxo variant (3.7%). The only orders that did not vary by diet were Flavobacteriales (one of the predominant groups at a mean 11.9%) and Sphingobacteriales (low abundance at mean 1.3%) (Fig. 3, Fig. 1B).

The proportion statistics confirmed the results described above, while also indicating additional relationships between the microbiomes (Fig. 4). The results that showed statistically significantly differences with concurrent large deviations in the proportions include: CEL vs. OAT: Selenomonadales and Lactobacillales for CEL and Rhodobacteriales and Clostridiales for OAT; OAT vs. PE-reg: Rhodobacteriales, Clostridiales and Bacteroidales for OAT and Rhizobiales, Lactobacillales and Enterobacteriales for PE-reg; OAT vs. PE-oxo: Flavobacteriales, Clostridiales, Bacteroidales and Rhodobacterales for OAT and Lactobacillales, Enterobacteriales and Entomoplasmatales for PE-oxo; OAT vs. PS: Rhodobacterales, Flavobacteriales, Clostridiales and Bacteroidales for OAT and Nitrosomonadales and Nitrospirales for PS; CEL vs. PE-reg: Selenomonadales and Bacteroidales for CEL and Pseudomonadales, Lactobacillales, Enterobacteriales and Rhizobiales for PE-reg; CEL vs. PE-oxo: Selenomonadales, Clostridiales, Bacteroidales and Flavobacteriales for CEL and Enterobacteriales, Lactobacillales and Rhodobacterales for PE-oxo; CEL vs. PS: Selenomonadales, Clostridiales, Enterobacteriales, and Bacteroidales for CEL and Nitrosomonadales, Nitrospirales, Lactobacillales and Planctomycetales for PS; PE-oxo vs. PE-reg: Rhodobacterales, Lactobacillales and Entomoplasmatales for PE-oxo and Pseudomonadales, Rhizobiales for PE-reg; PE-reg vs. PS: Rhizobiales, Entomoplasmatales and Clostridiales for PE-reg and Nitrosomonadales, Nitrospirales, Planctomycetales and Lactobacillales for PS; PE-oxo vs. PS: Rhodobacteriales, Lactobacillales, Enterobacteriales and Entomoplasmatales for PE-oxo, Planctomycetales, Nitrosomonadales, and Nitrospirales for PS.

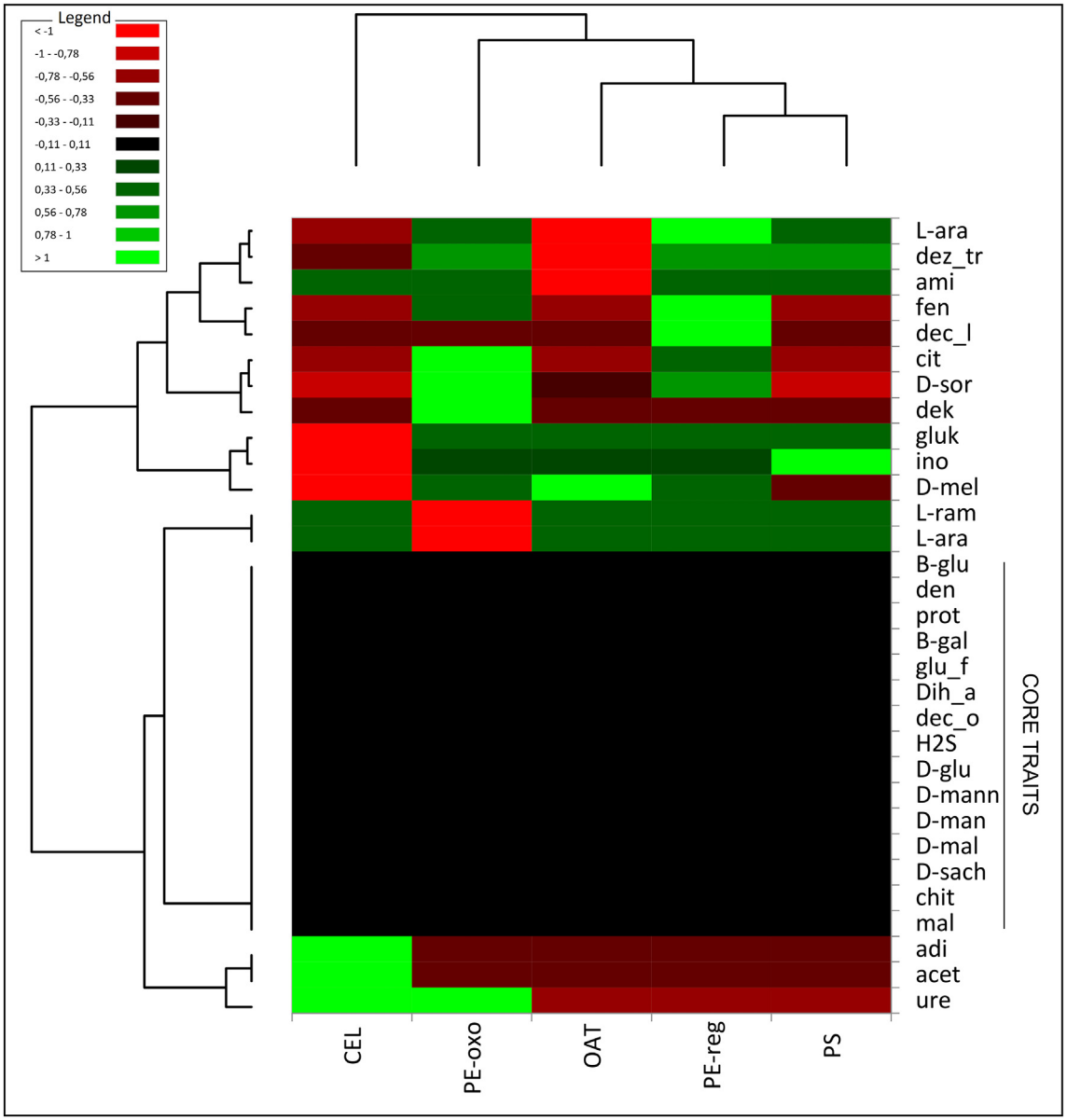

Fig. 2. Heat map of biochemical traits expressed by larval microbiomes. Variables were expressed as: +1 – light green (the highest deviation to mean) to -1 – light red (the lowest deviation to mean), black indicates no variability "0" or average activity for the specific parameter.

Principal component analysis (PCA) used to test for correlations between the diets and the examined parameters, such as the numbers of *Tenebrio molitor* at different stages of development, the concentration of proteins and fatty acids, and the presence of bacteria and fungi in the insects (Fig. 5a), showed that the first ordination axis, explaining 69.9% of the variance, was positively correlated with the PS diet, with the protein levels in the larvae and with a high load of the *amoA* gene responsible for nitrogen metabolism, and negatively correlated with presence of *Clostridium* bacteria in the digestive system of the larvae and the number of imagines. The second ordination axis, explaining 16.6% of the variance, was positively correlated with the lignocellulose diet and the content of MUFAs in *Tenebrio molitor*, as well as a high load of the *ureC* gene, and negatively correlated with PE-oxo and PE-reg diets, increased presence of *nifH* and *nirS* genes, as well as *Lactobacillus* bacteria fractions.

A PCA analysis of the relationship between the diet and individual compounds present in the mealworm larvae (Fig. 5b) showed that these two ordination axes explain 71.8% of the variance. The first ordination axis, explaining 44.6% of the variance, was positively correlated with the lignocellulose diet and the levels of acet, esteraseC4 and adipic acid as well as margaroleic acid, L-rhamnose, L-arabinose and leucin, as well as abundance of bacteria and *ureC*. The first axis was negatively correlated with presence of *nirS* and *nifH* genes, PE-reg and PE-oxo diets, as well as oleic acid, lysine decarboxylase, decanoic acid, D-melibiose, phenylacetic acid, trisodium citrate. The second ordination axis, explaining almost 30% of the variance, was positively correlated with the PS diet, as well as α-linoleic acid, Nitrospirales, Nitrosomonadales, and Planctomycetales. This axis was negatively correlated with A-ch and Rhodobacterales.

FTIR results showed clear differences in the diet only for PS. The profile of the feeding substrate (polystyrene) to frass was different for 5 of the 17 peaks identified. Frass differed from polystyrene by its absorbance at the length of 1067, 1233, 1540 and 1646 cm^{-1}, as well as by the lack of a common peak at 1601 cm^{-1} (Fig. 6).

4. Discussion

The present study shows that some of the diets caused significant changes in the biometry and chemical composition of the insects, the

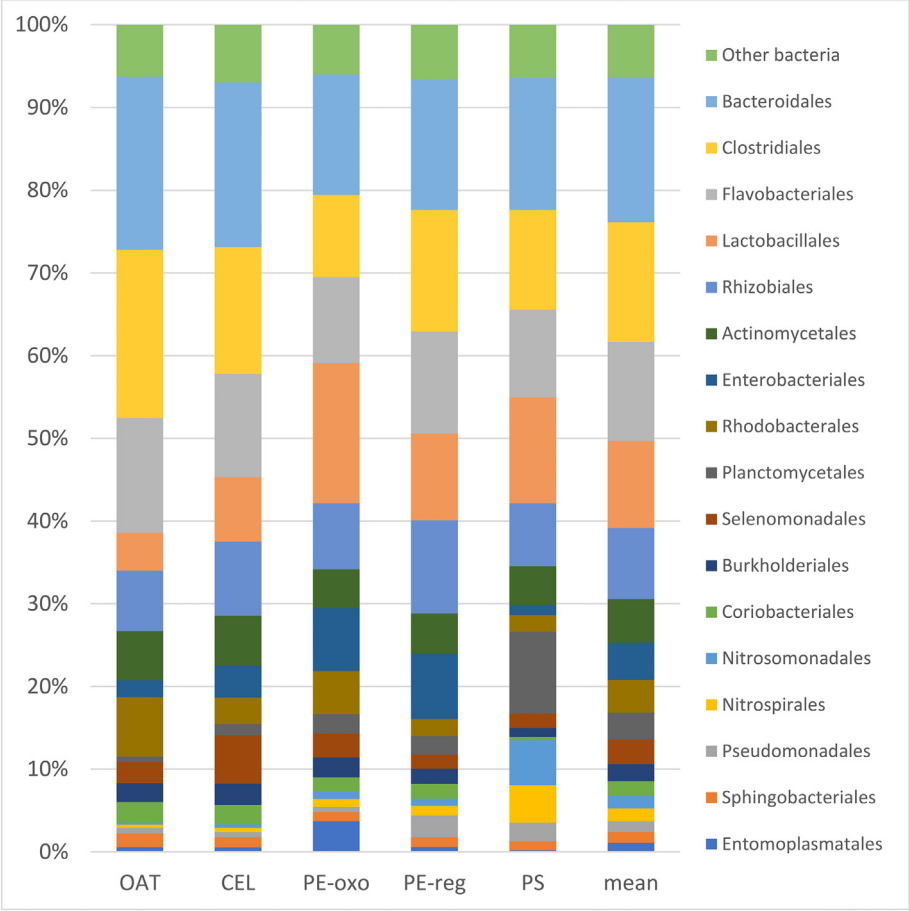

Fig. 3. The fraction of individual orders (not smaller than 1%) in the digestive system of *T. molitor*, by diet (OAT – oatmeal, CEL – cardboard, PE-oxo – oxo-degradable polyethylene, PE-reg – polyethylene regranulate, PS – polystyrene).

structure of their microbiome, and the biochemical activity in their digestive systems. Moreover, the results are partially divergent from the assumptions presented in our previous work dealing with the microbiome and enzymology of the mealworm digestive system (Przemieniecki et al., 2020). A comparative analysis of all the features covered by this study showed that all diets based on synthetic polymers are similar to each other, while the OAT and CEL are unique. This is consistent with the findings of Mancini et al. (2019), who showed that effect of the diet is related to the chemical composition of the substrates, although their experiments relied solely on substrates of natural origin. Other data matrix comparisons showed that the same is true only for a number of functional genes and, in part, for the 16S rRNA gene. This proves that some changes in insect digestive microbiomes caused by diet can lead to significant physiological changes in gastrointestinal microbial communities (Fig. 1).

In terms of biometrics (Table 1, Fig. 1A), it was observed that both the total mass of the larvae (more than 9 g) and the mass of a single larva (over 0.2 g) did not differ significantly in the PE-reg variant when compared to the control (OAT), which indicates that a diet based on this substrate has the least impact on the growth of biomass. On the other hand, other diets – especially CEL and PE-oxo –are not suitable and cause the larvae to starve. Interestingly, these results are comparable to those obtained by Mancini et al. (2019), who reported that a diet with a high protein concentration resulted in low final larval mass (0.14 g). Moreover, our observations regarding insect maturation indicate that the transitions between development phases and the size of the breeding in the PE-reg diet corresponded closest to the optimal diet, i.e. OAT. The rate of transition into later stages of development in the CEL group was significantly lower compared to the OAT and PE-reg variants and similar to PS and PE-oxo, where the larvae were able to transform into adult forms (Table 1, Fig. B), though the welfare of the population was disrupted. Similar results, showing the disruptive capacity of metamorphosis, were obtained by Yang et al. (2018a), who indicated that 6–11% of the above-mentioned observation may be optimal for PS consumption, while the 2nd generation of *T. molitor* degrades polystyrene much more efficiently. In the present study, full development was best achieved in the PE-reg diet, despite the high mortality of 25% (Fig. B) in the final phase of the experiment. The high rates of the latter were partially attributable to cannibalism, is a phenomenon well-known among *T. molitor* breeders and difficult to eliminate (Škrabalová and Vlk, 2011), occurring regardless of diet. The present study found that PE-reg plastic provided the most favorable development conditions for *T. molitor* to grow, which had not been observed before. Due to post-processing, regranulated polyethylene contains traces of non-polyethylene compounds, which may serve as an additional source of micronutrients. On the other hand, the increased cannibalism in the PE-reg variant indicates that diets based on synthetic waste need to be supplemented with food of natural origin (Appendix B). Larvae fed with synthetic polymers remain active by searching for food and consuming plastics. Although both grain diets and diets based on synthetic polymers result in some mortality, in the case of an optimal oatmeal diet, it is usually associated with the insect's natural life cycle, where an adult lays eggs and dies after metamorphosis. On the other hand, insects feeding on synthetic polymers may also have a lethal effect due to greater energy losses associated with digesting atypical food, as well as deficiencies in compounds necessary for life and development, as proven by Matyja et al. (2020). The first success in this area was realized by (Yang et al., 2018a) who, by feeding mealworm with a mixture of cereal bran with polystyrene and polyethylene, showed that a diet combining cereals and PS increases the survival rate of the larvae and enables the generation to fully grow. In their work on the

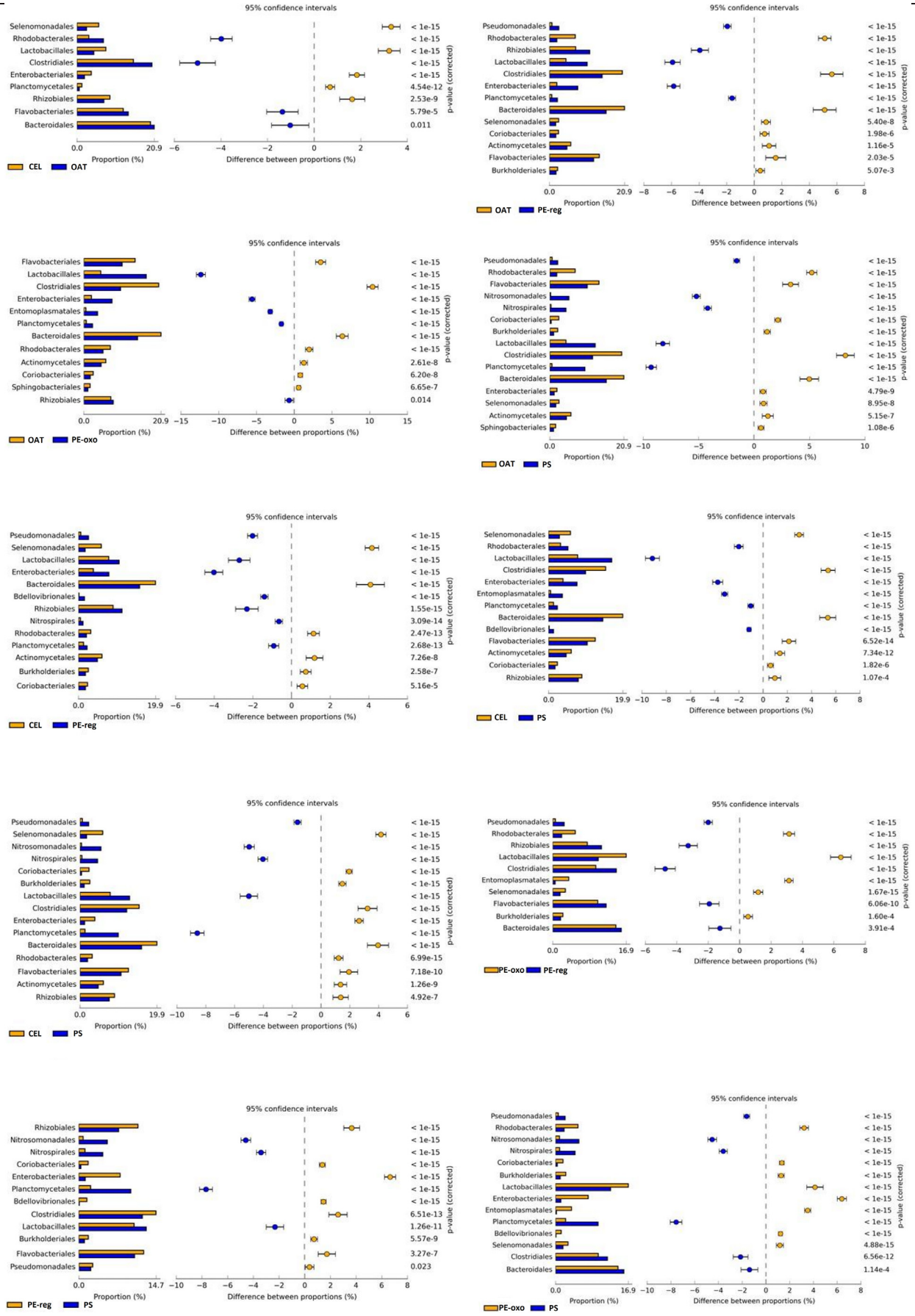

Fig. 4. The proportion statistics for significant bacterial orders inhabiting digestive system depending on diet.

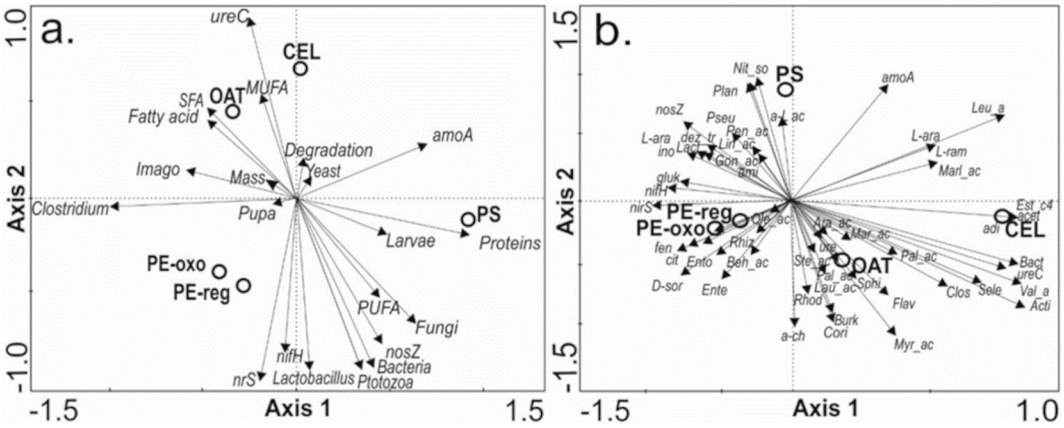

Fig. 5. Principal component analysis (PCA) showing relationships between mealworm diets a) plot based on population, insect biometry and qPCR results; b) plot based on microbiome and biochemical properties (abbreviations: OAT – oatmeal, CEL – cardboard, PE-oxo – oxo-degradable polyethylene, PE-reg – polyethylene regranulate, PS – polystyrene; containing fatty acids: MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids, SFA – saturated fatty acids, Lau_ac – lauric acid, Myr_ac – myristoleic acid, Pen_ac – pentadecanoic acid, Pal_ac – palmitic acid, Pal_ac – palmitoleic acid, Mar_ac – margaric acid, Marl_ac – margaroleic acid, Ste_ac – stearic acid, Ole_ac – oleic acid, Lin_ac – linoleic acid, α-L_ac – α-linoleic acid, Ara_ac –arachidic acid, Gon_ac – gondoic acid, Beh_ac – behenic acid; *amoA* – ammonium monooxygenase, *nifH* – nitrogenase reductase, *nirS* – nitrite reductase, *nosZ* – nitrous oxide reductase, *ureC* – urease; dez_tr – tryptophan deaminase, acet – production of acetoin, ure – urease, dec_l – lysine decarboxylase, D-sor – D-sorbitol, L-ram – L-rhamnose, D-mel – D-melibiosis, L-ara – L-arabinose, ino – inositol, ami – amygdalin, gluk – potassium gluconate, dek - decanoic acid, adi – adipic acid, cit – trisodium citrate, fen – phenylacetic acid; Ento – Entomoplasmatales, Sphi – Sphingobacteriales, Pseu – Pseudomonadales, Nit_sp – Nitrospirales, Nit_so – Nitrosomonadales, Cori – Coriobacteriales, Burk – Burkholderiales, Sele – Selenomonadales, Plan – Planctomycetales, Rhod – Rhodobacterales, Ente – Enterobacteriales, Acti – Actinomycetales, Rhiz – Rhizobiales, Lact – Lactobacillales, Flav – Flavobacteriales, Clos – Clostridiales, Bact – Bacteroidales).

use of *Tenebrio molitor* larvae to degrade polystyrene, Kosewska et al. (2019) also proved that the addition of an external nitrogen source in the form of DDGS (Distillers Dried Grains with Solubles) has a positive effect on the mass of individual larvae feeding on unconventional food such as polystyrene, which improves the condition of the entire breeding. The chemical composition of frass largely depends on the composition of the diet, but modern non-directional chemical methods not only identify the presence of polystyrene, but also determine the fate of metabolites formed after decomposition as described by the Tsochatzis et al. (2021a), Tsochatzis et al. (2021b). The results of the authors using GC-TOF-MS to determine the *T. molitor* metabolome proved that the degradation potential varies to some extent depending on the diet (e.g. different proportions of cereals to polystyrene), however the authors also proved that the presence of harmful impurities in both gut and in frass is negligible. Moreover, the authors found that the resulting substrates such as: 2,4,6-triphenyl-1-hexene and 1,3,5-triphenylcyclohexane, the volatiles acetophenone and cumyl alcohol, and 2,4-di-*tert* butylphenol, a non-intentionally added substance (NIAS), and long chain fatty acids, esters and amides indicate an intensive metabolic processes in the digestive tract of the larvae on a polystyrene diet (Tsochatzis et al., 2021a; Tsochatzis et al., 2021b). Our study partially confirms changes in the fatty acid profile and metabolic activity of the insect, however, in the case of polystyrene degradation, we have attached much greater importance to nitrogen utilization in the digestive system, as well as to a clear change in the proportion of protein in the total larvae body mass.

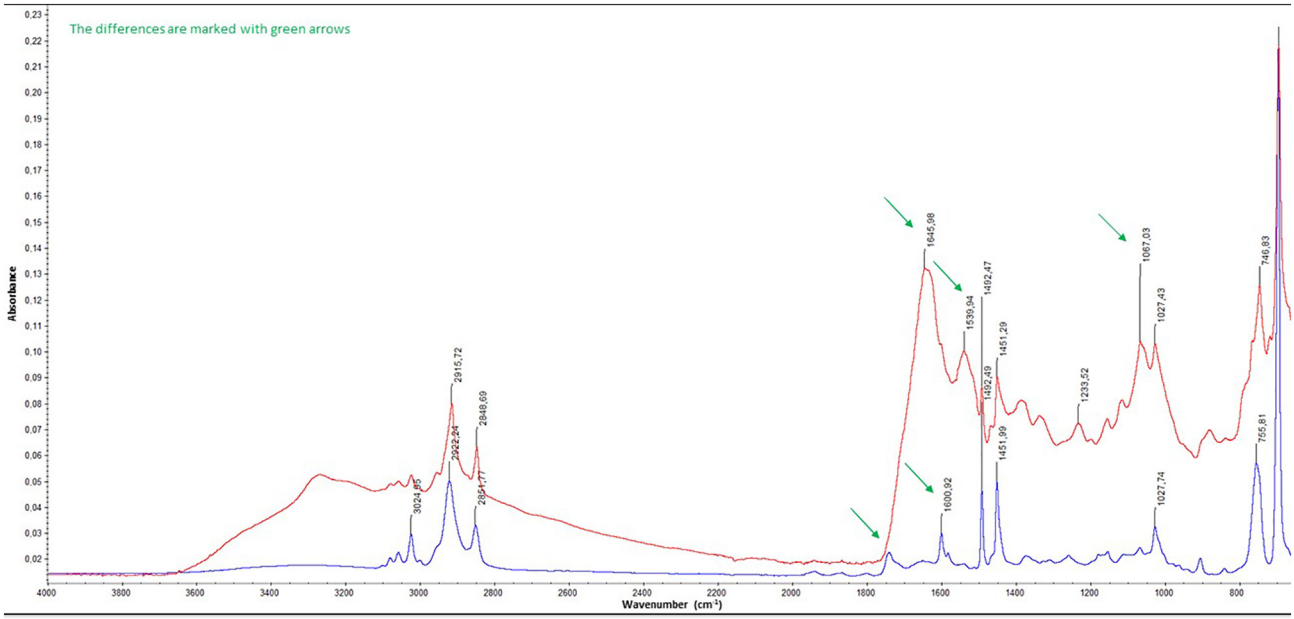

Fig. 6. FTIR analysis of mealworm frass (red line) obtained from polystyrene (PS; blue line) diet.

The results regarding protein and fat content in the larvae proved valuable for determining the condition of insects in the culture. The dissimilarity of OAT variants to the homogeneous group of CEL, PE-oxo and PE-reg (increased protein fractions and decreased fatty acid fractions), as well as the PS diet (highly increased protein and a clear reduction in fatty acids) may indicate dysfunction in fatty acid synthesis rather than protein synthesis. Moreover, calculating the ratio of protein to fat can provide an excellent biomarker of changes at the culture and physiological levels. The fatty acid profile was significantly different for the OAT diet compared to other diets, though the PS was also dissimilar from the polyethylene-based group (Table 3, Fig. 1D). Moreover, in terms of the quality of fatty acids, OAT contained the acids least suitable for human and animal consumption, PE was intermediate in quality, and PS had the highest total polyunsaturated fatty acids. Equally important, the food safety aspect should be taken into account and the resulting products must be tested for substances potentially dangerous for humans and animals. This is extremely important when insects were bred using unusual diets (Poma et al., 2017).

An alternative to the use of insect fats for consumption is environmental use. Each of the variants was suitable for the production of biodiesel. Moreover, insects grown on any diet are a reservoir of valuable protein, and technologies for the conversion and use of bioproducts are becoming increasingly popular (Wang et al., 2017; Zheng et al., 2013). It is surprising that the protein-forming capacity has been preserved despite the protein-deficient substrate, especially when it comes to the PS diet. Although on the one hand, there was a slight increase in cannibalism, on the other hand, numerous groups of microorganisms potentially responsible for nitrogen conversion may be involved in its redistribution and amino acid synthesis, as is the case with termites, whose diet is based on lignocellulose, practically devoid of nitrogen compounds (Cragg et al., 2015). The increase in protein after a PS-based diet has been further confirmed by Zielińska et al. (2021).

In this study, we have presented more detailed results of the biochemical analysis than in our previous work (Przemieniecki et al., 2020). These results show that the traits are relatively stable. Even so, half of them did vary depending on the diet (Fig. 2). We found that the microbiota in the CEL variant switched from glucose metabolism to adipic acid consumption combined with acetoin and urease production. PE-oxo was characterized by urease activity, but also by take-up of trisode citrate, D-sorbitol and decanoic acid. In contrast, OAT, PE-reg and PS exhibited a broad range of simple sugar metabolism. In contrast to OAT, the PE-reg diet resulted in wider take-up of a number of acids and sugars by the gut microbiome and digestive system, whereas the PS group showed increased inositol production capacity. The PE and PS groups shared a unique capacity for L-arabinose and amygdalin take-up and tryptophan deamination, activity which was low or absent in the CEL and OAT groups. It should be assumed that metabolism on plastic-based diets is related to or similar to the degradation of polymers in the *T. molitor* digestive system – alternatively, it may be an indicator of microbiota capable of degrading synthetic polymers. Pathak (2017) stated that the metabolism of a certain sugars (including arabinose) is partially related to the microbial degradation of plastics. As described by Yao et al. (2019), inositol metabolism in *Bactrocera minax* larvae is related to central metabolism and a strategy of reducing energy requirements of eukaryotic cells, nevertheless it is associated with fructose and mannose metabolism and also linked with high abundance of bacteria belonging to Leuconostocaceae, Lactobacillaceae, Bradyrhizobiaceae and Acetobacteraceae families. We obtained similar biochemical results in the present study and observed a high abundance of alphaproteobacteria and lactic acid bacteria, with the latter being particularly prolific.

Based on the results for the microbiomes by diet, it has been observed that relatively few orders of bacteria were completely replaced in the mealworm digestive system after switching to “plastic” diets. Nevertheless, there were several key changes in the make-up of the abundance of insect breeding depending on the diet. It seems that these subtle changes may be biomarkers of animal (insect) welfare (Fig. 3, Fig. 4). The orders of Rhodobacterales, Lactobacillales and Entomoplasmatales, and partly Enterobacteriales, were characteristic of the PE-oxo diet, whereas the OAT group had particularly high fractions of Flavobacteriales, Clostridiales, Bacteroidales and to a lesser extent Rhodobacterales. Several different orders of bacteria were found in increased proportions in the CEL-fed group, though only an increase in Selenomonadales was unique to this diet. Rhizobiales and Clostridiales appear to be microbiota characteristic of the PE-reg diet, which indicates the complex aerobic and anaerobic processes required to degrade this polymer. Nitrosomonadales, Nitrospirales, Planctomycetales and partially Lactobacillales are orders characteristic of the PS diet, which suggests that digestion of polystyrene is related to nitrogen metabolism and takes place in different parts of the digestive system (oxygenated and anaerobic). Entomoplasmatales is a class of bacteria commonly found in insects, often in the digestive system, though classified as an undesirable microbiota. The increased number of Entomoplasmatales in the PE-oxo variant indicates a pathogenic process, which may indicate that this diet is inadvisable. On the other hand, the increased proportion of Rhizobiales and Clostridiales in the PE-reg group, even though the microbiome is largely similar to PE-oxo, is a novel finding. Nevertheless, the dissimilarity of the PS variant (Fig. 3, Fig. 4, Fig. 1B) confirms our assumptions that this dietary change strongly affects the microbiome, a pattern is probably associated with nitrogen redistribution capacity (Przemieniecki et al., 2020). The present study demonstrated clear changes in the FTIR profile (Fig. 6). The lack of absorbance at 1067 cm^{-1} suggests no CC bond stretching. This points to partial depolymerization, whereas the differences in the distribution of peaks in the 2000–1500 cm-1 range may indicate a change in the structure of areas where double bond (C = C) stretching is commonplace, i.e. possible interference in the structure of the benzene molecules in polystyrene. These changes indicate a significant interference of the microbiome with the structure of polystyrene during digestion. Comparing this assumption with other research findings, it seems more likely that the stretches of double bonds results from the appearance of certain groups of bacteria or their metabolism, rather than a direct interference in the structure of the polymer, as described by Brandon et al. (2018). Nevertheless, other studies performed by Tsochatzis et al., 2021b have shown that a good way to determine the degradation potential of polystyrene based on frass is to analyze the lipophilic and polar fraction (long chain fatty acids, esters and amides) using GC-TOF-MS method.

Establishing a global correlation based on the PCA results proved to be a very complex task, but several major relationships were observed (Figure PCA1a). The CEL and OAT groups were found to contain high levels of urease subunit alpha (*ureC*) gene, MUFAs, SFAs, and fatty acids, which indicates the presence of bacteria capable of decomposing urea and higher levels of fatty acids, particularly MUFAs and SFAs (especially in OAT). High abundance of *amoA* (ammonium monooxygenase) is characteristic of CEL and PS groups, which indicates an increase in the nitrifying potential of the digestive system microbiota. In line with other analyses, the PS diet contributed to an increase in protein content in the insects and a slightly lower larval mass. The increase in nitrification may be an indicator of a deamination process taking place in the digestive system. A common feature for all plastic-based diets was the high content of fungi, bacteria, *nosZ* (nitrous oxide reductase), protozoa, *Lactobacillus* spp. and PUFAs, while the high load of bacterial *nifH* (nitrogenase reductase) and *nirS* genes was characteristic of polyethylene-based diets. It was also observed that the load of *Clostridium* spp. is not specific to PS. An interesting finding is the occurrence of *nosZ* (nitrous oxide reductase – denitrification), which indicates the potential ability to bind and convert reactive forms of nitric oxide into the molecular form of N_2. Previous studies of the N cycle in the digestive system provide only partial information and do not fully explain the process. The digestive system of insects is specific and has a functional microbiome structure partly different from that of warm-blooded organisms. In their review, Engel and Moran (2013), point to the prevalence of N-binding microorganisms in the digestive system of insects. The presence of these bacteria and protozoa suggests a capacity to produce amino acids using assimilated inorganic N (Cragg et al., 2015). Genes in insect gut microbiome and their role in nitrogen fixation was shown already by Aharon et al. (2013) and Bar-Shmuel et al. (2020). It was suggested that Enterobacteriales and Clostridiales bacteria could play a main role in nitrogen fixation (Aharon

et al., 2013; Bar-Shmuel et al., 2020). In this report, both Clostridiales and Enterobacteriales were found to form part of the gut microbiome regardless of diet. Moreover, as in our previous work (Przemieniecki et al., 2020), a high proportion of Planctomycetes was noted in the PS variant. Since bacteria of this order have the mechanisms for N-fixation and ammonia oxidation under both aerobic and anaerobic conditions, they facilitate nitrogen take up by the larvae (Delmont et al., 2018). The presence of Nitrosomonadales and Nitrospirales in PS variant, bacteria with many unique functions is puzzling. Nevertheless, this may suggest that the nitrification process plays an important role in the degradation of PS, which is also confirmed by the prevalence of bacteria carrying the *amoA* gene. Moreover, our previous work showed presence of bacteria of the genus *Nitrospira* most likely *N. defluvii* in *T. molitor* gut, which suggests a mixotrophic lifestyle with reverse tricarboxylic acid cycle, associated with the ability to take up CO_2 assimilation and a high capacity to adapt to limited substrate availability (Lücker et al., 2010). Nevertheless, the highest loads for *nifH* in PE-oxo, *nirS* in PE-reg and PE-oxo, and *nosZ* in all plastic-fed variants showing their direct relationship with the metabolism of degradation of synthetic waste has not been identified. Still, some correlations identified in the cluster analysis have been noted and described below.

The secondary results, produced by an analysis of the principal components (Fig. 5b), showed that the group of multi-traits characterized by a high abundance of Nitrosomonadales, Nitrospirales, Planctomycetales and α-linoleic acid, was closely linked to the PS diet, and slightly correlated with the consumption of amygdalin utilization, the levels of pentadecanoic acid, linoleic acid, gondoic acid content, as well as higher abundance of Pseudomonadales and *amoA*. The PE diets were distinguished by a group of features, which includes high capacity to consume phenylacetic acid, D-sorbitol, trisodium citrate, D-melibiose, decanoic acid; high activity of lysine decarboxylase; high levels of behenic acid; high fractions of Entomoplasmatales, Enterobacteriales, Rhizobiales; and high *nifH* and *nirS* gene abundance. A common group of parameters with high values for plastic-based diets were tryptophan deaminase activity, L-arabinose, inositol, glucose take-up, *nosZ* gene abundance and fraction of Lactobacillales. The remaining features characterized the OAT and/or CEL groups, though some parameters, such as C4 esterase activity, acetoin production and adipic acid take-up, were unique to the CEL diet. The PCA results showed multiple correlations between unique features and diet, mostly in line with the results of the analyzes conducted in this study. Each of the above-mentioned groups comprised a set of closely correlated multi-traits. The PE diets partially vary between one another in terms of the examined features and produce different results in terms of mealworm development. It is clearly visible that subtle changes in the physiology and structure of the microbiome are crucial for the welfare of insect breeding and the polyethylene waste degradation performance. Presumably, the structure of oxo-degradable polyethylene causes the formation of toxic forms of the polymer when degraded in the insect's digestive system. An assessment of oxidative stress and other toxicological studies would help to answer the question of whether this has an indirect or direct effect on the insect's body.

In conclusion, the results of our study show that there were two adaptations of *T. molitor* development resulting from the use of different diets. The first strategy focused on maintaining body mass and prolonging generation as long as possible, which was particularly evident in the PS variant. The second focused on preserving the reproductive cycle, meaning that the insect rapidly pupated to ensure the continuity of the species. The latter strategy was used in the control variant, in which the conditions were the closest to the insect's natural environment in terms of nutrition. A significant number of pupae also formed on the PE-reg substrate, indicating that the *T. molitor* breeding in this variant focused on producing the next generation. These observations are corroborated by studies of other scientists. Research on insect bioindicators, which include beetles of the Carabidae family, found that in areas contaminated with heavy metals (i.e. those less suitable for development), imagines incur higher physiological and genetic costs, but are nevertheless able to tolerate higher pollutant concentrations. The females grow to smaller sizes and start breeding faster than those from unpolluted habitats (Skalski et al., 2011). Much like the AHC, the PCA showed correlations, and partially explained changes in the microbiota and biochemistry of the digestive system and the entire body, depending on the diet. The presence of protozoa and intense biochemical changes, especially nitrogen, found in the digestive system point to an intensive digestive process and a suspected assimilation mechanism to facilitate survival on a polymeric monodiet. It should be noted that as the analyses included in this work encompass: the chemical composition of larvae, insect culture growth, FTIR spectrometry, changes at the biochemical and microbiome level – all excellent, though not yet fully developed, bioindicators. With the use of these parameters, we can determine the welfare of insects and their suitability for further processing. In addition, we can use mealworm larvae as a bioindicator of the state of the environment and identify the harmfulness of various environmental contaminants.

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Conceptualization - Sebastian Wojciech Przemieniecki, Agnieszka Kosewska; Data curation - Sebastian Wojciech Przemieniecki, Agnieszka Kosewska; Formal analysis - Sebastian Wojciech Przemieniecki, Olga Kosewska, Cezary Purwin, Krzysztof Lipiński; Funding acquisition - Sebastian Wojciech Przemieniecki, Agnieszka Kosewska; Investigation; Methodology - Sebastian Wojciech Przemieniecki, Sławomir Ciesielski; Project administration - Sebastian Wojciech Przemieniecki; Resources - Agnieszka Kosewska, Olga Kosewska; Software - Sebastian Wojciech Przemieniecki, Agnieszka Kosewska, Sławomir Ciesielski; Supervision - Sebastian Wojciech Przemieniecki, Cezary Purwin; Validation - Sławomir Ciesielski, Krzysztof Lipiński; Visualization - Sebastian Wojciech Przemieniecki, Olga Kosewska; Roles/Writing - original draft - Sebastian Wojciech Przemieniecki, Agnieszka Kosewska, Olga Kosewska; Writing - review & editing - Cezary Purwin, Krzysztof Lipiński.

Declaration of competing interest

The authors declare no conflict of interest.

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