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# Changes in the gut microbiome and enzymatic profile of *Tenebrio molitor* larvae biodegrading cellulose, polyethylene and polystyrene waste☆

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ABSTRACT

Recent studies have demonstrated the ability of mealworm (*Tenebrio molitor*) for plastic degradation. This study is focused on changes in microbiome structure depending on diets. Microbial community obtained from oat and cellulose diet formed similar group, two kinds of polyethylene formed another group, while polystyrene diet showed the highest dissimilarity. The highest relative abundance of bacteria colonizing gut was in PE-oxodegradable feeding, nevertheless all applied diets were higher in comparison to oat. Dominant phyla consisted of Proteobacteria, Bacteroides, Firmicutes and Actinobacteria, however after PS feeding frequency in Planctomycetes and Nitrospirae increased. The unique bacteria characteristic for cellulose diet belonged to Selenomonas, while *Pantoea* were characteristic for both polyethylene diets, *Lactococcus* and *Elizabethkingia* were unique for each plastic diet, and potential diazotropic bacteria were characteristic for polystyrene diet (*Agrobacterium*, *Nitrosomonas*, *Nitrospira*).

Enzymatic similarity between oatmeal and cellulose diets, was shown. All three plastics diet resulted in different activity in both, digestive tract and bacteria. The enzymes with the highest activity were included phosphatases, esterases, leucine arylamidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, chitinase, α-mannosidase and α-fucosidase. The activity of digestive tract was stronger than cultured gut bacteria. In addition to known polyethylene degradation methods, larvae may degrade polyethylene with esterase, cellulose and oatmeal waste activity is related with the activity of sugar-degrading enzymes, degradation of polystyrene with anaerobic processes and diazotrophs.



## 1. Introduction

Global waste production continues to increase due to higher levels of economic growth, improvement in living standards, increasing commercialization and consumption (Renou et al., 2008). Waste management poses a growing problem around the world. The generated waste is not decomposed naturally into organic matter and has to be managed by industrial means (Kostecka et al., 2014). Waste with prolonged biodegradation rate is particularly problematic. The modern era of synthetic plastics began in 1907 and it has multiplied the volume of plastic waste that enters the environment many-fold (Bartoczak, 2014). In 1989, the global production of plastics reached 64 million tons (Pielichowski et al., 1998), and it spiraled to 335 million tons by 2017 (PlasticEurope, Report 2017). According to UN data, the average consumer in highly developed countries produces around 100 kg of plastic waste, and this number continues to increase (Bartoczak, 2014). At present, the USA alone generates around 33 million tons of plastic waste each year (Jordan, 2015). These figures give cause for concern because inappropriately managed waste has a negative impact on the environment. Waste pollution poses a threat to both terrestrial and aquatic ecosystems (Moore et al., 2011). Seas and oceans are polluted with immense amounts of polymer-based waste which occupy an area equivalent to the area of Australia

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and weigh more than 100 million tons (Heimowska, 2016). Plastic waste is not the only problem facing humanity because all types of waste, including cellulose-based, have to be managed. Alternative methods of waste management are being developed to address this pressing issue.

Waste production cannot be avoided, which is why various technologies have been proposed for managing waste that decomposes at a faster rate as well as problematic waste. One of such methods relies on insect activity. Mealworm larvae are well suited for waste processing on account of their voracious appetite, resistance to adverse environmental conditions and rapid weight gain (Siemianowska et al., 2013). Mealworms are omnivores that are capable of digesting food that is not found in the natural environment, including polyethylene, polystyrene and cellulose waste, moreover it has been confirmed in worldwide collective works (Nukmal et al., 2018; Yang et al., 2018, Vigneron et al., 2019). Researchers hypothesize that gut bacteria play an important role in mealworms' ability to adapt to different foods (Wang and Zhang, 2015; Brandon et al., 2018). Mealworm larvae can feed on polystyrene (Yang et al., 2015a,b) which is digested in a multi-stage process. Polystyrene is chewed by larvae, which increases its specific surface area and promotes contact with the microorganisms and extracellular enzymes in the larval digestive tract. Crushed polystyrene reaches the intestines and is mixed with gut microbiota which secrete enzymes that catalyze the decomposition of polystyrene into fragments with a low molecular weight. Polystyrene is processed and carbon dioxide is produced during the reaction. The process relies on synergistic interactions between microorganisms and the mealworm host. Polystyrene residues and other undigested waste are excreted with feces (Yang et al., 2015a). Cellulose-based diets are digested in a similar manner. According to Willis et al. (2010), mealworms are one of the few insect species capable of breaking down cellulose. The digestive tract of *Tenebrio molitor* is highly differentiated and adapted to digesting various types of food. Mealworm gut microbiota are able to decompose lignocelluloses which are found in resistant cellulose waste (Huang et al., 2012).

**The aim of this study** was to: i) determine the changes in microbial community inhabiting gastrointestinal tract of mealworm larvae depending on the diet; ii) determine changes in the enzymatic activity of gut bacteria in *Tenebrio molitor* larvae fed various types of waste; iii) identify alleged bacterial groups capable of utilization polyethylene, polystyrene and cellulose wastes.

## 2. Materials and methods

### 2.1. Sampling design

The study was performed on selected mealworm larvae from the collection of the Department of Entomology, Phytopathology and Molecular Diagnostics of the University of Warmia and Mazury in Poland. Mealworm larvae were fed five substrates: cardboard (CEL), polystyrene (PS), oxo-degradable polyethylene (PE-oxo), polyethylene regranulate (PE-reg) and oatmeal waste (OAT) as the control substrate. Glass containers with a volume of 1 l were filled with equal amounts of the substrate (100 ml), and 100 larvae were placed inside every container. After two months of breeding mealworm, the larvae were collected for further analysis, and the waste substrate and frass were weighed to determine the effectiveness of waste degradation. Every substrate variant was analyzed in three replications.

In this study, a common approach was used to prepare environmental samples for molecular analysis (Hermann-Bank et al., 2013; Tkacz et al., 2018). Larval digestive tracts were excised (100 mg) after two months of feeding. DNA was isolated from the collected material with the QIAamp PowerFecal DNA Kit (Qiagen, Germany) and concentrations of each samples were quantified by fluorometric quantitation on Quantus™ Fluorometer (Promega, Germany).

### 2.2. Load of bacteria

The bacterial genomic DNA concentration was determined in the TaqMan assay with the use of the Maxima Probe Real-time PCR Master Mix 2X (Thermo Fischer Scientific, Waltham, USA), 2 μl of extracted DNA, 500 nM of every primers (BAC338F 5′-ACTCC-TACGGGAGGC-3′ and BAC805R 5′-GACTACCAGGGTATCTAATC C-3′) and 200 nM of the probe BAC516F 5′FAM-TGCCAGCAGCC5GCGG TAATA-TAMRA3' (Yu et al., 2005). The final reaction volume was 20 μl. The reaction profile was as follows: initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s and annealing/elongation at 60 °C for 60 s. Each of the five tested variants was analyzed in two replications. As standard genomic DNA from *Bacillus subtilis* A9 was used.

### 2.3. Amplicon sequencing and bioinformatics analysis

The microbial communities colonizing 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 technique. The primers were developed by adding Illumina adapter overhang nucleotide sequences to the PCR primers given by Klindworth et al. (2013). Amplicons were indexed using the Nextera® XT Index Kit according to the manufacturer's instructions. DNA was sequenced in Illumina MiSeq in 2 × 250 paired-end mode. Sequencing results 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). 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). 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.

### 2.4. Biochemical properties of microbiome

The enzymatic activity and carbon source utilization of larval digestive tracts and gut microbiota were determined in the API ZYM enzymatic test and API 20 NE, 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 the TissueLyser LT bead mill (Qiagen, Germany) at 30 oscillations for 5 min. The resulting suspension was diluted 1:10 and used in API tests.

The initial procedure for separating gut microbiota was identical to that deployed during the isolation of digestive tract cells. Gut microorganisms were homogenized at 40 oscillations for 10 min, the homogenate was centrifuged at 500×*g* to remove digestive tract cells, and the supernatant was used in the API tests.

### 2.5. Statistical analysis

The counts and species composition of microorganisms were analyzed. The species diversity of the analyzed microorganisms was determined with the use of Shannon diversity index (H′), Pielou's evenness index (J′) and Simpson's diversity index (D). The relationships between the administered substrate, the microorganisms identified in the digestive tracts of mealworm larvae and their enzymatic activity were determined by principal component analysis (PCA) and agglomerative hierarchical clustering (AHC). The differences between mean values were determined by one-way analysis of variance (ANOVA) at a significance level of 0.05. Homogeneous groups were identified with the use of Duncan's test. The results were processed statistically and interpreted graphically in XLSTAT (Addinsoft), Biodiversity Pro (McAleece et al., 1997), Statistica v. 12.5 (StatSoft, 2012) and Canoco v. 4.5 programs.

## 3. Results and discussion

Application of mealworm larvae are effective for degrading polystyrene and other plastics however their efficiency could be various depending on the type of waste and other factors (Yang et al., 2018; Brandon et al., 2018; Vigneron et al., 2019). In our previous studies on PE and PS degradation, a decrease in substrate mass after 70 days of mealworm larvae feeding was demonstrated. In the case of a control feed consisting of oatmeal, this was a loss of 31.7%, while the weight of the polystyrene substrate decreased by 12.2% (Kosewska et al., 2019), and the weight of the polyethylene substrate decreased by 16.6%, which is clear proof that the larvae were nourished by the tested substrate. In this study we also showed that mealworm larvae degraded waste. After taking mass of frass into account, the degradation efficiency after 2 months of cultivation were: OAT-10.56, CEL-5.2, PE-reg-5.92, PE-oxo-6.22, PS-7.88%, respectively. In addition, we observed that cannibalism occurs regardless of the diet.

The taxonomic classification of bacteria isolated from the digestive tracts of mealworm larvae produced 98102 reads representing 972 species (Table 1). The highest number of individuals and species was noted in the digestive tracts of mealworm larvae fed cardboard and biodegradable plastic. The values of Shannon's diversity index (H′) and Pielou's evenness index (J′) were highest in mealworm larvae fed polyethylene regranulate. The value of H′ is generally higher in smaller communities with a more stable structure. Simpson's diversity index (D), also referred to as the dominance index, gives more weight to more common and abundant species than singleton species in a sample. In this study, the highest value of D was noted in larvae fed the control substrate (oatmeal).

The concentration of bacterial DNA was highest (62 ng DNA $g^{-1}$) in larvae fed oxo-degradable polyethylene (PE-oxo). The results noted in the above variant differed significantly from oatmeal (OAT) and cardboard (CEL) variants where the content of bacterial DNA was estimated at 28 ng DNA $g^{-1}$ of the sample. The content of bacterial DNA in larvae fed polyethylene regranulate (PE-reg) and polystyrene (PS) was approximately 40% higher than in OAT and CEL variants, but the noted differences were not statistically significant (Fig. 1).

**Table 1**
Number of reads and species and the diversity indicators of microorganisms isolated from the digestive tracts of mealworm larvae fed different substrates.

| Description of microbiome | Substrate[a] | | | | |
|---|---|---|---|---|---|
| | OAT | CEL | PE-oxo | PE-reg | PS |
| Total individuals (reads) | 15906 | 25908 | 23075 | 17524 | 15689 |
| Total species | 519 | 610 | 588 | 551 | 557 |
| Mean individuals | 16.381 | 26.682 | 23.764 | 18.047 | 16.158 |
| Standard Error | 3.876 | 5.745 | 4.448 | 3.382 | 3.397 |
| Shannon's H′, log base 2.718 | 3.974 | 4.153 | 4.187 | 4.263 | 4.133 |
| Pielou's J′ | 0.636 | 0.648 | 0.657 | 0.675 | 0.654 |
| Simpson's D | 0.057 | 0.047 | 0.036 | 0.036 | 0.045 |

[a] OAT – oatmeal waste, CEL – cardboard, PE-oxo – oxo-degradable polyethylene, PE-reg – polyethylene regranulate, PS – polystyrene.

At phylum level, an analysis of differences in the structure of microbial communities between feeding variants revealed that Proteobacteria, Bacteroidetes, Firmicutes were the eudominant phyla, whereas Actinobacteria was the dominant phylum (Fig. 2). In most cases, the proportions of bacterial phyla differed significantly between larval diets (Figure S1). The proportion of Proteobacteria was lower only in variant PS (34.5% vs. the average of 37%). The proportion of Bacteroides (average of 25.7%) was highest in variant OAT (29%) and lowest in variants PE-oxo and PS (23%). Firmicutes was characterized by equal proportions in all variants, excluding PE-oxo where it was clearly higher (26% vs. the average of 23.7%). The proportions of the remaining bacterial phyla were higher in variant PS: Planctomycetes (8.1% vs. the average of 2.7%), Nitrospirae (3.7% vs. the average of 1.2%), Verrucomicrobia (2.1% vs. the average of 1.5%) and Aquificae (0.5% vs. the average of 0.1%). Variant PE-oxo was characterized by a significantly higher proportion of Terenicutes (3.2%) which did not exceed 0.5% in the remaining feeding variants (Fig. 2, Figure S1).

Alphaproteobacteria, Bacteroides, Clostridia, Flavobacteria and Bacilli were the dominant classes in nearly all feeding variants. The proportions of bacteria in larvae fed various substrates were more differentiated at class level than at phylum level. The dominant class was Alphaproteobacteria (24.4% on average) whose frequency was higher in variants OAT and CEL (28%) than in the remaining variants (approx. 22%). Bacteria of the class Bacteroides (14.3% on average) were distributed similarly to Alphaproteobacteria, whereas the proportions of Clostridia (12%) were clearly higher in variant OAT (16%) and lowest in variants PE-oxo and PS (approx. 9.5%). The class Bacilli was most widely distributed across the analyzed feeding variants, and its proportions were highest in variants PE-oxo (15%) and PS (11%) and lowest in variant OAT (4%). Gammaproteobacteria were characteristic of both PE variants (approx. 10% vs. the average of 7.6%). Actinobacteria were somewhat more abundant in variants OAT and CEL. It should be noted

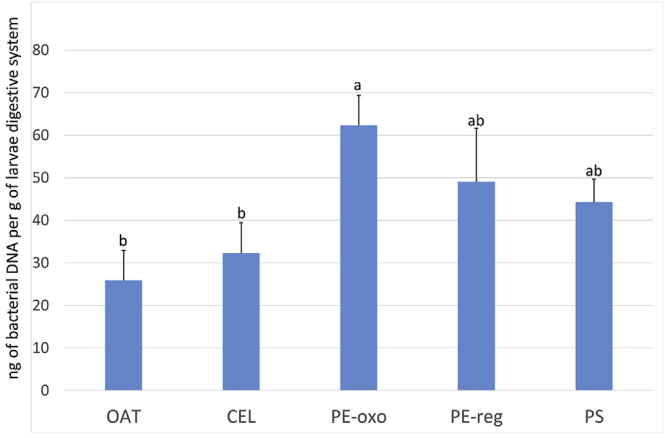


**Fig. 1.** Content of bacterial DNA in the digestive tracts of *T. molitor* larvae fed different substrates estimated by qPCR. Abbreviations: OAT – oatmeal waste, CEL – cardboard, PE-oxo – oxo-degradable polyethylene, PE-reg – polyethylene regranulate, PS – polystyrene; Values marked with the same letters (a or b) do not differ significantly within the enzyme ($p \leq 0.05$).

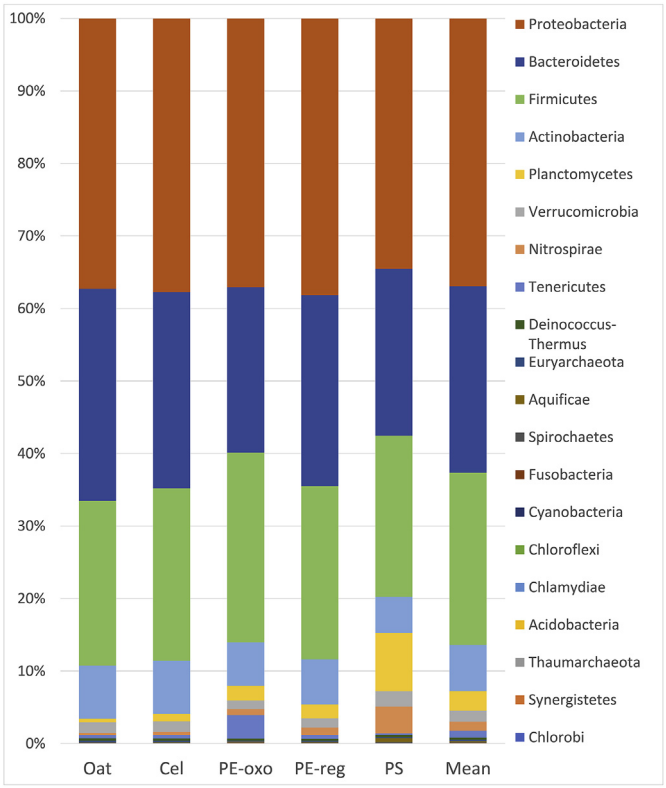


**Fig. 2.** A comparison of microbiomes based on the bacterial phyla identified in the digestive tracts of mealworm larvae fed different substrates. Abbreviations: OAT – oatmeal waste, CEL – cardboard, PE-oxo – oxo-degradable polyethylene, PE-reg – polyethylene regranulate, PS – polystyrene.

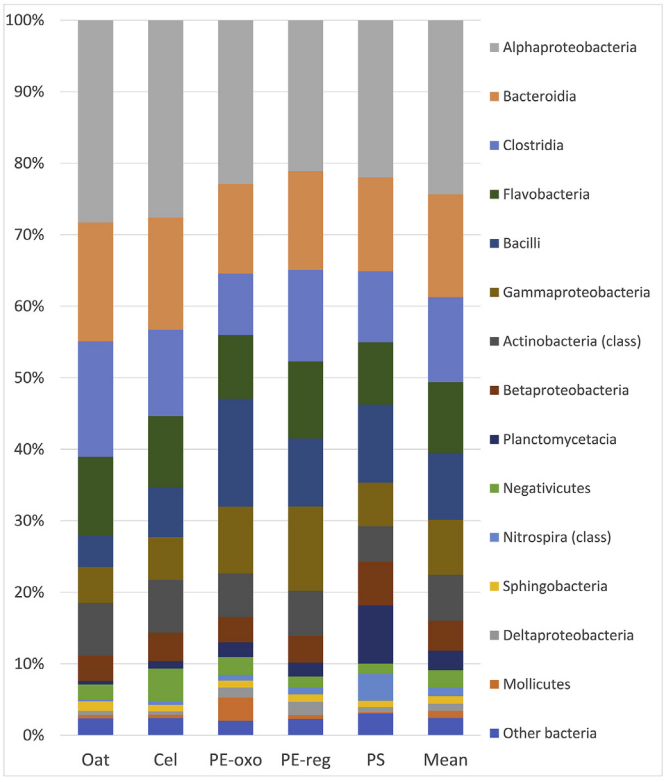


**Fig. 3.** A comparison of microbiomes based on bacterial classes identified in the digestive tracts of mealworm larvae fed different substrates. Abbreviations: OAT – oatmeal waste, CEL – cardboard, PE-oxo – oxo-degradable polyethylene, PE-reg – polyethylene regranulate, PS – polystyrene.

that the proportions of Planctomycetes (8%), Betaproteobacteria (6%) and Nitrospira (4%) were clearly higher in larvae fed polystyrene. Other distinctive bacterial classes were Negativicutes (5%) in variant OAT and Mollicutes (3%) in variant PE-oxo (Fig. 3, Figure S2).

The proportions of bacterial genera differed across substrate variants, but selected genera were characterized by stable frequencies. The most prevalent genera were *Parabacteroides* (16.4% on average), *Clostridium* (9.3%) and *Agrobacterium* (5.0%). In larvae fed oatmeal, the eudominant genera were *Parabacteroides* (20%) and *Clostridium* (11%). The above variant was also characterized by relatively high proportions of *Paracoccus* spp. (7.4%), *Agrobacterium* spp. (4.8%), *Symbiobacterium* spp. (4.4%), *Ruminococcus* spp. (3.9%) and *Lactobacillus* spp. (3.6%). The proportions of *Parabacteroides* (19%) and Clostridium (11%) were similar in variants CEL and OAT. In these variants, *Selenomonas* (6.4%) and *Agrobacterium* (6%) were the dominant genera. In variant PE-oxo, the proportions of *Parabacteroides* spp. (13%), *Clostridium* spp. (6.3%), *Paracoccus* spp. (4.9%) and *Ruminococcus* spp. (2.3%) were lower than in the control variant (OAT). The most prevalent genera in variant PE-oxo were *Pantoea* (8.0%), *Lactobacillus* (6.1%), *Paracoccus* (4.9%), *Lactococcus* (4.5%), *Spiroplasma* (4.0%) and *Elizabetkinga* (2.5%). Larvae fed polyethylene regranulate (PE-reg) were characterized by a lower proportion of the eudominant genus *Parabacteroides* (15%) and a clear decrease in the proportions of *Selenomonas* spp. (1.7% vs. the average of 3.1%). Bacterial genera *Clostridium* (11%), *Pantoea* (7.3%), *Agrobacterium* (5.5%), *Rhizobium* (5.3%) and *Elizabetkinga* (3.4%) were prevalent in variant PE-reg. Genera *Parabacteroides* (16%), *Lactococcus* (8.4%), *Clostridium* (7.8%) *Elizabetkinga* (6.6%), *Nitrosomonas* (6.1%) and *Agrobacterium* (5.7%) were characteristic of larvae fed polystyrene.

The proportions of genera *Parabcteroides*, *Clostridium*, *Lactobacillus* and *Agrobacterium* differed across the evaluated feeding variants, but these genera were always highly prevalent (at least subdominant) in the communities of larval gut bacteria. Bacterial genera whose proportions differed significantly between the experimental variants and the control variant (OAT) were *Lactococcus* in variants PE-reg, PE-oxo and PS, *Rhizobium* in variant PE-reg, *Nitrosomonas, Nitrospira* and *Elizabethkinga* in variant PS, *Pantoea* in both PE variants, *Selenomonas* in variant CEL, and *Acinetobacter* in variants PE-reg and PS. The frequency of *Enterococcus* spp. was clearly lower in the control variant (OAT) than the remaining variants. Another noteworthy observation was the highly significant decrease in the frequency of *Clostridium* spp. in variant PE-oxo (6.2% vs. the average of 9.3%) (Table 2).

The results of the metagenomic analysis demonstrated certain trends in the prevalence of bacterial groups across the tested feeding variants. Larvae fed oatmeal and cellulose were colonized by similar bacterial communities, and certain similarities were also observed in both polyethylene variants. The microbiota isolated from larvae fed polystyrene differed most considerably from the remaining bacterial groups. The predominance of *Parabacteroides* spp. and *Clostridium* spp. indicates that these bacteria rely on anaerobic mechanisms to break down plastics that are difficult to decompose (Toczyłowska-Mamińska et al., 2018).

An analysis of the overall results of enzymatic activity (Table 3) revealed that the concentrations of enzymes secreted by digestive tract homogenates were higher (21 pmol on average) than those secreted by bacteria alone (13 pmol on average). A qualitative analysis demonstrated that trypsin was the only inactive enzyme in bacteria, whereas the digestive tract did not produce α-

**Table 2**
Structure of bacterial genera in mealworm larvae fed different substrates[a].

| Phylum | Class | Order | Genus | OAT | CEL | PE-oxo | PE-reg | PS | Mean |
|---|---|---|---|---|---|---|---|---|---|
| Actinobacteria | Actinobacteria (class) | Actinomycetales | *Arthrobacter* | 1.4 | 1.2 | 1.0 | 1.0 | 0.8 | 1.1 |
| | | | *Leucobacter* | 2.4 | 2.1 | 1.6 | 1.5 | 1.7 | 1.9 |
| | | | *Rothia* | 1.2 | 1.2 | 1.0 | 0.9 | 1.1 | 1.1 |
| | | Coriobacteriales | *Collinsella* | 2.4 | 2.1 | 1.6 | 1.7 | 0.2 | 1.6 |
| Bacteroidetes | Bacteroidia | Bacteroidales | *Bacteroides* | 1.7 | 1.4 | 0.9 | 1.1 | 1.2 | 1.3 |
| | | | *Porphyromonas* | 1.1 | 1.5 | 1.1 | 0.9 | 0.9 | 1.1 |
| | | | *Parabacteroides* | 20.2 | 18.6 | 13.3 | 14.6 | 15.5 | 16.4 |
| | Flavobacteria | Flavobacteriales | *Chryseobacterium* | 0.7 | 0.8 | 1.3 | 0.9 | 1.4 | 1.0 |
| | | | *Elizabethkingia* | 0.7 | 0.8 | 2.5 | 3.4 | 6.6 | 2.8 |
| | | | *Flavobacterium* | 2.2 | 2.0 | 1.5 | 1.7 | 1.6 | 1.8 |
| | Sphingobacteria | Sphingobacteriales | *Terrimonas* | 1.4 | 1.0 | 0.7 | 1.1 | 0.8 | 1.0 |
| Firmicutes | Bacilli | Lactobacillales | *Enterococcus* | 0.5 | 2.7 | 2.6 | 2.6 | 1.7 | 2.0 |
| | | | *Lactobacillus* | 3.6 | 3.9 | 6.1 | 4.4 | 3.1 | 4.2 |
| | | | *Lactococcus* | 0.1 | 0.8 | 4.5 | 3.0 | 8.4 | 3.4 |
| | Clostridia | Clostridiales | *Clostridium* | 11.3 | 10.8 | 6.2 | 10.5 | 7.8 | 9.3 |
| | | | *Ruminococcus* | 3.9 | 3.6 | 2.3 | 3.0 | 3.3 | 3.2 |
| | | | *Symbiobacterium* | 4.4 | 0.5 | 0.3 | 0.3 | 0.4 | 1.2 |
| | Negativicutes | Selenomonadales | *Selenomonas* | 2.7 | 6.4 | 3.0 | 1.7 | 1.7 | 3.1 |
| Nitrospirae | Nitrospira (class) | Nitrospirales | *Nitrospira* | 0.4 | 0.5 | 1.0 | 1.2 | 5.1 | 1.6 |
| Proteobacteria | Alphaproteobacteria | Rhizobiales | *Agrobacterium* | 4.8 | 6.0 | 3.1 | 5.5 | 5.7 | 5.0 |
| | | | *Rhizobium* | 1.9 | 2.6 | 1.9 | 5.3 | 1.8 | 2.7 |
| | | Rhodobacterales | *Paracoccus* | 7.4 | 3.0 | 4.9 | 1.5 | 1.7 | 3.7 |
| | Betaproteobacteria | Nitrosomonadales | *Nitrosomonas* | 0.2 | 0.4 | 0.9 | 0.9 | 6.1 | 1.7 |
| | Gammaproteobacteria | Enterobacteriales | *Pantoea* | 1.3 | 0.3 | 8.0 | 7.3 | 1.1 | 3.6 |
| | | Pseudomonadales | *Acinetobacter* | 0.6 | 0.6 | 0.5 | 2.7 | 2.5 | 1.4 |
| Tenericutes | Mollicutes | Entomoplasmatales | *Spiroplasma* | 0.5 | 0.6 | 4.0 | 0.6 | 0.2 | 1.2 |

[a] OAT – oatmeal waste, CEL – cardboard, PE-oxo – oxo-degradable polyethylene, PE-reg – polyethylene regranulate, PS – polystyrene.

**Table 3**
Activity of hydrolytic enzymes in the gut bacterial communities and digestive tracts of mealworm larvae fed different substrates[a].

| Enzyme | Concentration [p mol] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Digestive tract | | | | | Gut bacteria | | | | |
| | OAT | CEL | PE- oxo | PE- reg | PS | OAT | CEL | PE-oxo | PE-reg | PS |
| Alkaline phosphatase | 33.3a[b] | 33.3a | 33.3a | 30.0a | 30.0a | 3.3b | 3.3b | 11.7b | 6.7b | 13.3b |
| Esterase (C4) | 6.7b | 5.0b | 6.7b | 13.3a | 10.0a | 3.3c | 10.0a | 5.0b | 5.0b | 3.3c |
| Ester lipase (C8) | 5.0 ab | 6.7 ab | 6.7 ab | 10.0a | 6.7 ab | 1.7b | 1.7b | 3.3 ab | 1.7b | 1.7b |
| Lipase (C14) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Leucine arylamidase | 33.3 | 33.3 | 33.3 | 36.7 | 36.7 | 30.0 | 30.0 | 23.3 | 23.3 | 26.7 |
| Valine arylamidase | 3.3 | 3.3 | 3.3 | 3.3 | 3.3 | 1.7 | 1.7 | 0.0 | 0.0 | 0.0 |
| Cystine arylamidase | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Trypsin | 3.3 | 0.0 | 1.7 | 1.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| α-chymotrypsin | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.7 | 1.7 | 1.7 | 1.7 | 0.0 |
| Acid phosphatase | 40.0a | 40.0a | 40.0a | 40.0a | 40.0a | 33.3 ab | 30.0b | 30.0b | 33.3 ab | 36.7 ab |
| Naphthol-AS-BI phosphohydrolase | 40.0a | 40.0a | 40.0a | 36.7 ab | 40.0a | 36.7 ab | 33.3 ab | 23.3 ab | 20.0b | 30.0 ab |
| α-Galactosidase | 5.0a | 3.3 ab | 3.3 ab | 3.3 ab | 5.0a | 0.0b | 0.0b | 0.0b | 0.0b | 0.0b |
| β-Galactosidase | 40.0 | 40.0 | 40.0 | 40.0 | 40.0 | 40.0 | 36.7 | 33.3 | 36.7 | 36.7 |
| β-Glucuronidase | 40.0a | 40.0a | 40.0a | 40.0a | 40.0a | 36.7 ab | 33.3b | 33.3b | 26.7b | 26.7b |
| α-Glucosidase | 40.0a | 36.7a | 33.3a | 36.7a | 33.3a | 26.7 ab | 13.3bc | 15.0bc | 8.3c | 16.7bc |
| β-Glucosidase | 26.7a | 23.3a | 18.3 ab | 23.3a | 23.3a | 16.7 ab | 16.7 ab | 13.3 ab | 10.0 ab | 8.3b |
| N-Acetyl-ß-glucosaminidase | 33.3 ab | 30.0a-c | 30.0a-c | 30.0a-c | 30.0a-c | 36.7a | 26.7a-c | 20.0bc | 26.7a-c | 16.7c |
| α-Mannosidase | 30.0a | 26.7 ab | 26.7 ab | 26.7 ab | 26.7 ab | 13.3bc | 13.3bc | 8.3c | 10.0c | 8.3c |
| α-Fucosidase | 30.0a | 33.3a | 30.0a | 30.0a | 30.0a | 8.3b | 10.0b | 6.7b | 6.7b | 6.7b |
| Mean | **22** | **21** | **20** | **21** | **21** | **15** | **13** | **12** | **11** | **12** |
| Sum of characters | **17** | **16** | **17** | **17** | **16** | **16** | **16** | **15** | **15** | **14** |

[a] OAT – oatmeal waste, CEL – cardboard, PE-oxo – oxo-degradable polyethylene, PE-reg – polyethylene regranulate, PS – polystyrene.
[b] Values marked with the same letters (a, b or c) do not differ significantly within the enzyme ($p \leq 0.05$).

chymotrypsin, and its production was marginal in the isolated bacterial consortium. Lipase (C14) and cystine arylamidase were completely inactive. The enzymes with the highest average levels of activity in all variants were β-galactosidase (lactase; 38 pmol), acid phosphatase (36 pmol), β-glucuronidase (35 pmol), naphthol-AS-BI-phosphohydrolase (34 pmol) and leucine arylamidase (31 pmol). N-acetyl-ß-glucosaminidase (chitinase; 28 pmol) and α-glucosidase (maltase; 26 pmol) were characterized by relatively high overall activity levels. Average levels of activity were noted in alkaline phosphatase (less than 20 pmol), α-fucosidase and α-mannosidase (19 pmol each) as well as β-glucosidase (cellulase; 18 pmol). The activity of the remaining active enzymes did not exceed 6 pmol.

A comparison of the detailed results of the analysis of enzymatic activity revealed that alkaline phosphatase was significantly more active in the digestive tract of larvae fed PE-oxo and PS (above 30 pmol), and its activity in bacteria exceeded 10 pmol. Smaller differences were observed in acid phosphatase whose activity was significantly lower (by 10 pmol) only in the bacterial community isolated from variants OAT and PE-oxo. The activity of naphthol-AS-

BI-phosphohydrolase was determined at nearly 40 pmol in the digestive tract, and it was only somewhat lower in the isolated bacterial consortia.

Similar levels of C4 esterase activity were noted in the digestive tract and in bacterial communities. In the case of digestive tract its activity was the highest in the PE-reg and PS variant, while in the case of the microbial community in the CEL variant. The activity of C8 esterases was significantly lower (by 8 pmol) only in the bacterial communities isolated from variant PE-reg. Despite differences in the activity of proteolytic enzymes leucine arylamidase, valine arylamidase and α-chymotrypsin, their activity levels did not differ significantly between diets and objects (gut/bacteria).

Oxidase activity differed between variants, and the variations observed in bacteria were diet-dependent. Significant differences in the concentration of α-galactosidase were observed between objects, and they were more pronounced in the digestive tracts of larvae fed OAT and PS. The concentration of α-fucosidase was significantly lower (by approx. 23 pmol) in bacteria than in the digestive tract of larvae fed every type of diet. Significant differences in α-mannosidase activity were observed between the control variant (OAT) (30 pmol in the digestive tract; up to 13 pmol in bacteria) and variants PE-oxo, PE-reg and PS (estimated decrease of 18 pmol in bacteria). Alpha-glucosidase was the only enzyme whose activity in bacterial communities differed significantly between feeding variants. The first of the above enzymes was characterized by significantly lower activity (by 19 pmol) in bacterial consortia in variants CEL, PE-oxo and PS; its activity in variant PE-reg was reduced by 29 pmol, and it was significantly lower in the experimental variants than in the control variant. Significant differences were not observed in oxidases, including β-galactosidase and β-glucuronidase which were most active in this group of enzymes, nor in β-glucosidase and N-acetyl-ß-glucosaminidase (Table 3).

The results of the PCA evaluating enzyme activity in the homogenates of larval digestive tracts revealed variations in the distribution of hormone secretions across the tested feeding variants. The typical diet of mealworm larvae (OAT) was correlated with the majority of sugars, including α-mannosidase, β-glucosidase, N-acetyl-α-glucosaminidase and β-glucosidase. The cellulose diet was correlated with α-fucosidase and enzymes catalyzing the breakdown of phosphorus bonds: alkaline phosphatase and naphthol-AS-BI-phosphohydrolase. The presence of C4 and C8 esterases and leucine arylamidase in the digestive tract was correlated with the diet composed of polyethylene regranulate (PE-reg). Variant PE-oxo was correlated with the y-axis which described more than 30% of the observed variation, but specific enzymes were not bound by clear correlations with variant PE-oxo or variant PS (Fig. 4a).

The results of the PCA evaluating the enzymatic activity of bacteria isolated from the digestive tracts of mealworm larvae revealed the presence of correlations between most enzymes from all groups (proteins, fats and carbohydrates) with the x-axis which described nearly 60% of the observed variation. Similarly to the homogenates of the digestive tracts of *T. molitor* larvae, variant OAT was correlated with the x-axis. Variant CEL was correlated with the y-axis which described more than 20% of the observed variation. Variant CEL was clearly correlated with the secretion of esterase C4 and α-chymotrypsin. Variants PS and PE-oxo were clearly correlated with the secretion of esterase-lipase C8 and enzymes catalyzing the breakdown of phosphorus bonds, alkaline phosphatase and acid phosphatase. Unlike the evaluated homogenates, none of the bacterial enzymes isolated from the digestive tracts of mealworm larvae were correlated with variant PE-reg (Fig. 4b).

The results of enzymatic analyses indicate that digestive tract lysates (with symbionts) are far more active than the microorganisms alone. In a qualitative analysis, the studied objects differed only in two enzymes, which suggests that some enzymes can be produced by digestive tract cells as well as by symbiotic bacteria. The above does not rule out the possibility that enzymes can be produced only by symbiotic bacteria in *T. molitor*. The pool of enzymes produced by the digestive tract and the isolated bacterial community was relatively homogenous (without significant differences within the studied objects), which points to high

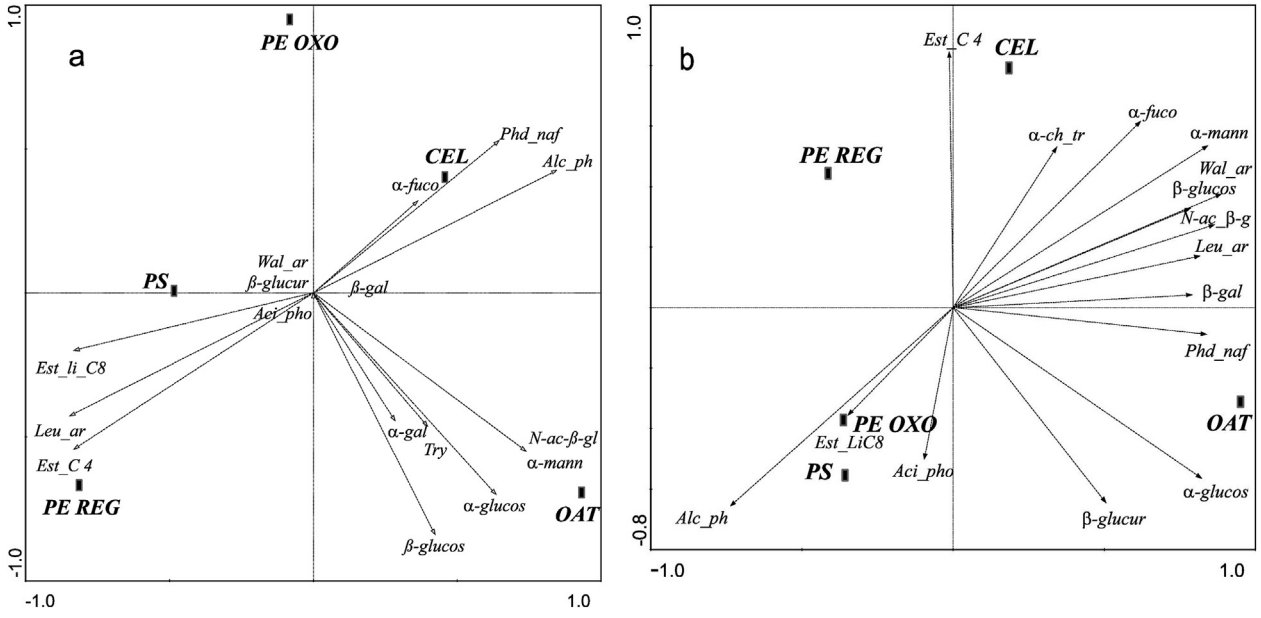


**Fig. 4.** The correlations between diet and enzyme activity in the digestive tract (a) and the bacterial community (b) determined in PCA. (abbreviations: OAT - oatmeal waste, CEL - cardboard, PE-oxo - oxo-degradable polyethylene, PE-reg - polyethylene regranulate, PS - polystyrene; Alc_ph - alkaline phosphatase, Est_C 4- C4 esterase, Est_li_C8 - C8 esterase lipase, Lip_C14 - lipase (C14), Leu_ar - leucine arylamidase, Wal_ar - valine arylamidase, Ary_cys - cystine arylamidase, Try – trypsin, α-ch_tr - α-chymotrypsin, Aci_pho - acid phosphatase, Phd_naf - naphthol-AS-BI-phosphohydrolase, α-gal - α-galactosidase, β-gal - β-galactosidase, β-glucur - β-glucuronidase, α-glucos - α-glucosidase, β-glucos - β-glucosidase, N-ac_β-gl - N-acetyl-ß-glucosaminidase, α-mann - α-mannosidase, α-fuco - α-fucosidase).

enzymatic stability across the tested diets. However, significant differences in activity between the studied objects indicate that the application of selected microorganisms for degrading polymer-based waste could be problematic. Further and more detailed research is needed to address this issue.

The most active enzymes were the three enzymes which catalyze the breakdown of phosphorus bonds and which are indicative of rapid P and C transformations. The presence of these enzymes indicates that organic phosphorus compounds are effectively transformed into inorganic soluble phosphates that are available for living organisms (Hoppe and Ulrich, 1999; Hoppe, 2003; Margalef et al., 2017). The results of the PCA revealed the strongest correlations between the activity of alkaline phosphatase and acid phosphatase and polystyrene in gut bacteria. It should also be noted that the activity of C8 ester lipase in both the digestive tract and the bacterial community was elevated in variant PE-reg. The presence of a correlation between C8 ester lipase and polyethylene could be indicative of a possible route of polyethylene degradation by *T. molitor*. According to Ramya et al., (2016), the digestive tract of *Plutella xylostella* butterflies is colonized by numerous bacterial species that produce esterases. The cited authors demonstrated that esterase-producing bacteria (*Pseudomonas, Enterococcus, Bacillus, Pantoea agglomerans*) were responsible for the degradation of indoxacarb insecticide. Esterases and lipases have been found to degrade polyethylene. In other studies, the activity of depolymerizing enzymes was accompanied by high levels of phosphatase activity, and such correlations are typically observed in environments where the distribution of biogenic elements initiates numerous reactions (Cooper et al., 1991; Hoppe and Ulrich, 1999; Scully et al., 2013; Margalef et al., 2017).

The hierarchical cluster analysis revealed similarities in the enzymatic activity and structure of the bacterial community (Fig. 5). The studied variants differed in enzymatic activity. In the digestive tract, clade I composed of variants PS and PE-reg and clade II composed of variants PE-oxo, CEL and OAT were separated by an estimated distance of 0.7x. In the bacterial community, variant PE-oxo belonged to clade I and was more similar to variants PS and PE-reg, and the distance between clades exceeded 2.5x. The hierarchical cluster analysis of microbiomes in the tested variants produced three clades: clade I – variant PS, clade II – variants PE-reg and PE-oxo, and clade III – variants CEL and OAT. The distance between variant PS and the remaining variants was 12.2x, the distance between variants in clade II was 6.4x, and the distance between variants in clade III was 3.6x. The enzymatic activity of the bacterial community and microbiome structure were grouped identically in hierarchical cluster analysis. However, enzymatic activity in variant PE-oxo was more similar to variant CEL than variant PE-reg in the digestive tract (Fig. 5).

A detailed analysis of the potential role of the evaluated microbiota revealed that in variant PS, the digestive tract of *T. molitor* contained an additional source of carbon as well as atmospheric nitrogen fixed by bacteria of the orders Rhizobiales, Nitrospirales and Nitrosomonadales. Nitrification and anammox reactions can take place in the digestive tracts of mealworms fed diets deficient in nitrogen compounds. These reactions can promote the absorption of available forms of nitrogen, as suggested by the significantly higher counts of *Nitrospira* sp. and *Nitrosomonas* sp. bacteria. The noted variations in the activity of esterases indicate that these enzymes are probably involved in polystyrene degradation. This observation also suggests that bacteria can effectively decompose polystyrene, in particular oxo-degradable polystyrene, in a symbiotic relationship with the larval digestive tract. In addition to utilizing polymer wastes, *T. molitor* are also diazotrophs that distribute nitrogen in symbiotic interactions, which makes them potential candidates for the production of cheap protein sources for food production and other commercial usage. Similar observations have been made in termites and wood-damaging pests that feed on lignocellulosic monosubstrates and other substances that are difficult to decompose (Engel and Moran, 2013; Cragg et al., 2015). A review of the literature on bacterial functions in a wide range of insect hosts revealed that microorganisms play a variety of functions, subject to insect species and its specific requirements (Engel and Moran, 2013). Studies of insects other than *T. molitor* demonstrated that Proteobacteria and selected species of Actinobacteria (such as *Rhodococcus* sp.) supply insects with food (production of amino acids) and are predominant in the digestive tract. The digestive tract of termites is colonized by a microbial community consist several hundred species of symbiotic microorganisms with a stable structure and frequency. In insects of the genus *Nasutitermes*, bacterial phyla with constant proportions, including Spirochaetes, Fibrobacters, Proteobacteria, Bacteroidetes and Acidobacteria, are responsible for cellulose degradation, nitrogen fixing and cycling, fermentation and nutrient supply (Hongoh et al., 2005, 2008; Desay and Brune, 2012). In *Reticulitermes speratus* termites, Protozoa and Spirochaetes bacteria with a well-defined structure decompose lignocellulose (Warnecke et al., 2007;

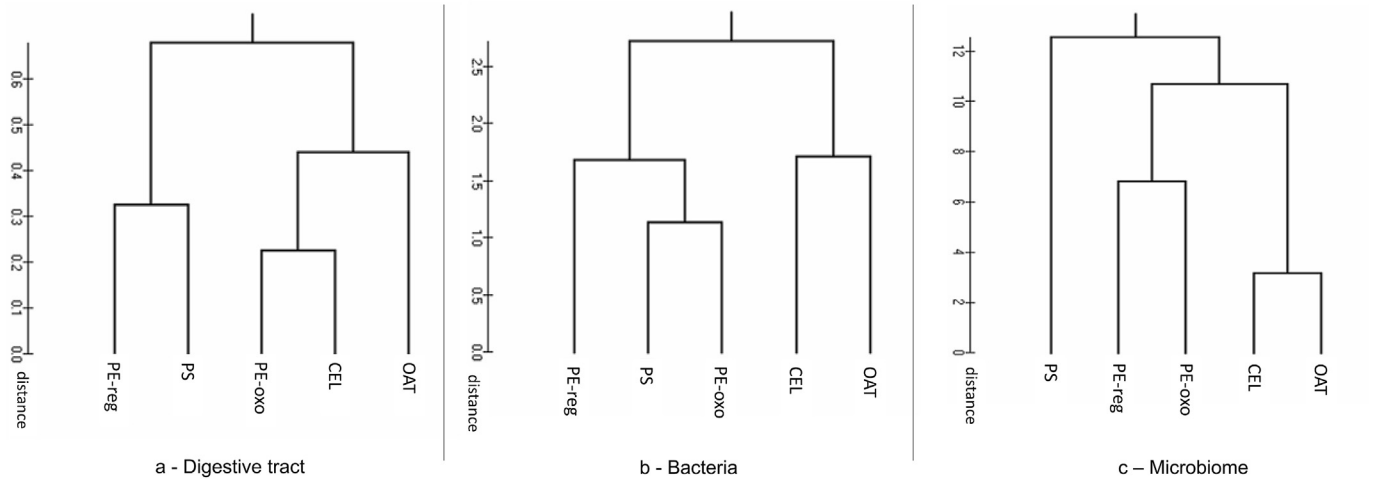


**Fig. 5.** The results of a hierarchical clustering analysis indicating differences in a) enzymes active in the digestive tract, b) enzymes produced by bacteria, c) microbiome structure across the tested feeding variants.

Köhler et al., 2012) and have various industrial applications (Brune, 2014, Auer et al., 2017).

According to Brandon et al. (2018) the predominant bacterial taxa in mealworm larvae fed bran, polystyrene and polyethylene were *Spiroplasma* spp., *Cronobacter* spp. and *Enterococcus* spp. Larvae fed both plastics were colonized mainly by *Citrobacter* spp. and *Kosakonia* spp. (Enterobacteriaceae). Polyethylene-based diets increased the proportions of *Sebaldella termitidis* and *Brevibacterium* spp., whereas polystyrene-based diets increased the proportions of *Listeria* spp. and *Nitrospira defluvii*. Larvae fed bran were colonized mainly by *Clostridium* spp. and *Bacillus* spp. Nevertheless, some of research indicated that were some changes in gut, "whole larvae" and frass microbiome and traits between larvae growing in similar condition however different batch (Osimani et al., 2018; Yang et al., 2018).

In this study, bacteria of the order Rhizobiales were the accompanying microbiota with a relatively stable frequency. According to the literature, most bacteria that enter into symbiotic relationships with insects belong to the orders Burkholderiales, Pseudomonadales, Rhizobiales, Verrucomicrobiales and Xanthomonadales (Sapountzis et al., 2015; Russell et al., 2009), as well as *Wolbachia* and Entomoplasmatales (Sapountzis et al., 2015). In the present study, the average proportion of potentially N-fixing bacteria in the total bacterial community was determined at 7%.

Symbionts can promote indirect reassimilation of $NH_3$ and atmospheric nitrogen to promote insect survival in nitrogen-deficient environments. The potential activity of symbiotic bacteria (nitrogenase) is dependent on the availability of trace elements that make up the enzyme's active site, including iron, sulfur, vanadium and manganese (Igarashi and Seefeldt, 2003). This observation suggests that the availability of nitrogen, trace elements and water in nutrient-deficient habitats is a limiting factor which can lead to cannibalism in order to provide access to substances that facilitate nitrogen assimilation by symbionts.

## 4. Conclusion

This is the first study to compare the effects of metabolism of various types of polymer waste (two types of polyethylene, polystyrene and cellulose) in *T. molitor* larvae on the microbiome and hydrolytic enzymes in the larval digestive tract. Enzymatic activity was generally stable across feeding variants, but esterase activity increased in larvae fed both types of polyethylene. Next-generation sequencing supported the identification of other bacterial groups that participate in waste degradation. Larvae fed every analyzed diet were colonized by *Parabacteroides* spp. and *Clostridium* spp. anaerobic bacteria that decompose hemicellulose and polyethylene under natural conditions. The identification of bacteria of the genus *Pantoea* in larvae fed both polyethylene diets, *Lactococcus* and unique genera such as *Elizabethkingia* bacteria in larvae fed all plastic diets, and potential diazotrophic bacteria in the microbiota characteristic of the polystyrene diet (*Agrobacterium* spp., *Nitrosomonas* spp., *Nitrospira* spp. and the less abundant phylum Verrucomicrobia) was an important achievement of this study. The above observation suggests that gut microbiota in mealworm larvae are capable of synthesizing proteins from atmospheric nitrogen.

## Conflicts of interest

The authors declare that they have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113265.

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