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A comprehensive assessment of microbiome diversity in *Tenebrio molitor* fed with polystyrene waste☆

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ABSTRACT

Recently it was demonstrated that mealworm (*Tenebrio molitor*) larvae consume and biodegrade polystyrene. Thus, in this study a breeding investigation with various types of polystyrene was performed to follow the changes in the gut microbiome diversity. Polystyrene used for packaging purposes (PSp) and expanded polystyrene (EPS) were perceived as more favorable and attacked more frequently by mealworms compared to raw polystyrene (PS) and material commercially available for parcels (PSp). Although our studies showed that larvae could bite and chew selected materials, they are not able to degrade and use them for consumption purposes. In a next-generation sequencing experiment, among all samples, seven classes, *Gammaproteobacteria*, *Bacilli*, *Clostridia*, *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria* and *Flavobacteria*, were indicated as the most abundant, whereas the predominant genera were *Enterobacter*, *Lactococcus* and *Enterococcus*. Additionally, we isolated three bacteria strains able to use diverse types of bioplastic as a sole carbon source. The strains with biodegradable activity against bioplastic were identified as species of the genera *Klebsiella, Pseudomonas* and *Serratia*. The presence of a bacterial strain able to degrade bioplastic may suggest a potential niche for further investigations.

1. Introduction

Nowadays plastic materials are an integral and very important part of the global economy and humans' daily life. The extensive use of this material creates a plastic pollution problem that is seen as a major threat to ecological systems as a result of uncontrolled dumping of plastic wastes (Halpern et al., 2008; Shah et al., 2014; Urbanek et al., 2018). Due to the long-term persistence and extreme resistance to degradation (Gajendiran et al., 2016), once plastic wastes enter the environment, they remain there indefinitely. The vast majority of garbage comes from the packaging sector, where one of the predominantly used resin types in production is polystyrene [PS], which represents 7% of all plastics. PS is a synthetic aromatic polymer which is manufactured from the monomer styrene. It has been used in many types of product, including general purpose polystyrene (GPPS), high impact polystyrene (HIPS), PS foam and expanded polystyrene foam (EPS). PS not only causes environmental pollution, but also due to its toxicity and recalcitrant compounds has an impact on human health problems and ecosystem changes (Ho et al., 2018). Due to the enormous threat of conventional plastic to the environment, more attention is paid to biodegradable plastics such as poly(butylene succinate) (PBS), poly(butylene succinate-co-butylene adipate) (PBSA) and polycaprolactone (PCL). In contrast to the petrochemical plastics, PBS degrades naturally into water and CO_2, which makes it environmentally friendly (Xu & Guo, 2010). PBSA is produced similarly to PBS with adipic acid as an additive, which makes it more flexible (Vroman & Tighzert, 2009). Degradation of PCL may take place in soil, water, and compost by microorganisms (Tokiwa et al., 2009).

In spite of the fact that plastic materials are durable and resistant to damage, it has been reported that packed foods can be attacked by storage insects. The ability to chew plastic items is a result of having sharp mandibles (Hassan et al., 2016). Moreover, it has been reported that some bugs can live on a diet of plastic due to their ability to digest some plastics that are chemically similar to natural polymers. The ability to eat plastic has been proved in the case of the greater wax moth *Galleria mellonella* (Bombelli et al.,

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2017), red flour beetle *Tribolium castaneum* and the lesser grain borer *Rhyzopertha dominica* (Hassan et al., 2016). Recently it was shown that the larvae of *Tenebrio molitor*, commonly known as mealworms, are able to chew and eat EPS (Yang et al., 2015a). However, the tendency to eat plastic might be associated with the digestive activity of intestinal microorganisms, which would help them with the digesting process. Symbiotic microorganisms have been found widely in the guts, body cavities, or cells of a wide array of insects (Hosokawa et al., 2006). These microorganisms aggregate into a large symbiotic organ called the bacteriome (Douglas, 1998). It is well known that the gut-inhabiting microorganisms play a significant role in the digestive process to provide essential nutrients for the host insects (Akman et al., 2002; Douglas, 1998; Suzuki et al., 2013). Symbionts are also involved in detoxification of toxic compounds and production of volatile substances (Genta et al., 2006). Based on this it can be presumed that microorganisms play a significant role in degradation of plastic compounds in the insects' guts.

The aim of this study was to determine whether mealworms are able to digest different types of PS (raw material, processed and expanded polystyrene, and polystyrene used for packaging) and investigate the changes in gut microbiome diversity depending on type of the provided PS. In order to assess the effectiveness of the biodegradation process we studied the properties of different PS types to find out which one is the most suitable for biodegradation. We also compared the effectiveness of biodegradation of PS by mealworm larvae and imagines on the basis of mass changes of both studied PS types and mealworms. Additionally, we registered changes of total lipid, sugar and carbohydrate content in bodies of *T. molitor* according to the feeding type. Furthermore, we assessed the microbial fluctuations and the abundance of bacteria in the digestive systems of *T. molitor* between larvae and imagines. Finally, we identified three bacterial strains able to degrade bioplastics such as PBSA, PBS and PCL.

2. Material and methods

2.1. Larvae and imagines

Larvae and imagines of *T. molitor* were derived from the inbreeding "Cricket farm" in Lublin (Poland). We determined 6 parallel groups, 30 specimens each, according to their similar mass within the range of 0.02–0.04g. We prepared seven different variants:

- PS: larvae **fed** with raw polystyrene, 1 mm thick plates were prepared;
- PSr: larvae **fed** with processed polystyrene by extrusion, injection molding and grinding. The described set of actions was performed ten times;
- PSp: larvae **fed with** commercially available material for parcels;
- EPS: larvae **fed with** commercially available insulation material – expanded polystyrene;
- S: starved larvae;
- I: imagines (adults);
- C: (control) larvae **fed** with oatmeal *ad libitum.*

Mealworm breeding conditions: temperature 28 °C ± 1 °C, humidity 78–80%. We conducted the experiment for 21 days. The mass of studied mealworms was assessed on the 1st and 21st day of the experiment. Next, a group of mealworms was collected.

The guts consisting of three-part structure – foregut, midgut, and hindgut – of the larvae and imagines were separated using a scalpel in sterile conditions. The guts were dissected from 10 individuals in every variant.

2.2. Materials

Polystyrene granules for preparation of raw polystyrene were obtained from the joint-stock company Krakchemia S.A. (Poland), brand name SYNTHOS PS GP 137. Before the first processing cycle, a raw polystyrene was dried in an oven (70 °C, 3 h). PBSA (Bionolle 3020MD) and PBS (Bionolle 1020MD) pellets were obtained from Showa Denko K.K. (Japan). PCL pellets were purchased from TRESNO (Poland). Sarkosil NL, for emulsions preparation, was purchased from Sigma-Aldrich (Germany).

2.3. Media

The media used for isolation of bacteria from the digestive system of *T. molitor*, preservation of the isolated strains, verification of their biodegradable abilities and evaluation of the total number microorganisms were prepared as follows.

For isolation and estimation of the total number of microorganisms in mealworm guts, nutrient agar (NA) (Merck, Germany) and potato count agar (PCA) (BIOCORP, Poland) were used. To select bacteria with the ability to degrade bioplastic we used minimum medium (FMM). The composition as described previously (Kitamoto et al., 2011) with slight modification was 0.2% NaH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.02% KH_2PO_4, 0.1% yeast extract and 0.1% emulsified plastic (PS, PCL, PBSA, PBS) as a sole carbon source (Urbanek et al., 2017). The suspensions of isolated and crushed guts were stored in 70% glycerol at −80 °C.

2.4. Preparation of polymer emulsion

Plastic 0.5% emulsions (PS, PCL, PBSA, PBS) were prepared as described below (modified from Uchida et al., 2000): 2 g plastic pellets was dissolved in 40–60 ml of dichloromethane, consequently 2 ml of 2% Sarkosyl NL and 100 ml of distilled water were added. The obtained solution was sonicated for 10 min (Sonics Vibra Cell VCX500), in order to evaporate dichloromethane the mixture was stirred at 80 °C for 2 h in a draft chamber. The polymer emulsion was completed with 400 ml of distilled water. The pH was 7.0 and it was adjusted by adding KOH.

2.5. Preparation of materials

We used a press molding process to form plates with dimensions 100 × 100 × 0.1 mm for all studied material variants in order to do physico-mechanical assessment. Before the consumption test press molding was also applied for PS and PSr, although PSp and EPS did not undergo further processing, they were only divided into smaller pieces.

One-screw extruder (HAAKE Rheomex 252), injection molding machine (BOY 35A) and grinder (Wanner) was applied for processing polystyrene. Standard and recommended by manufacturer values of temperature for PS thermoplastic processing were set. The following extrusion process temperatures on the extruder's zones were applied: 205 °C, 220 °C, 210 °C, 200 °C (die zone temperature), whereas 200–250 °C was the injection molding temperature. We set the injection pressure profile from 700 bars down to 550 bars, he cycle time interval was set to 2 s. LabTech LP-20B was used for press molding under the circumstances: temperature 220 °C, plasticization time 60 s, pressure applied: 50 bar and actual process time 120 s.

Melt Flow Junior (CEAST) apparatus was used to asses melt flow ratio under the following conditions: load (2.16 kg), temperature (210 °C), cutting time (30 s), preheating time (120 s). We repeated

each measurement at least three times (until we achieved the difference among three measured points not greater than 10%).

We used Shore D (for PS and PSr) and Shore A scale (for PSp and EPS samples) to assess hardness. We apply 1 kg load to the tip end.

Gloss was measured at 60° (semi-gloss surface) using Multi Gloss 268 Plus (Konica Minolta) was used for contact angle measurements. All materials were kept 48 h at 50% RH before being tested in static conditions on an Advex Instrument. A 2.5 μL droplet of solvent was applied on the film surface. We recorded the evolution of the droplet shape every second by a video camera, and then we use the image analysis software to determine the contact angle values. At least 10 average measurements were defined as the water contact angle value. We determined an error as a standard deviation of the mean value and it was depicted as error bars.

2.6. Total number of bacteria in the various groups of mealworms

The total number of bacteria was determined by the microbial plate count method. The homogenate suspension of larvae guts was prepared as follows: each gut isolated from each larva was separately homogenized by crushing and suspending in 1 ml of 0.85% sterile saline water. After homogenization for 30 s in a vortex, homogenate samples were used as a seed stock of microorganisms for isolation. The series of tenfold dilution were prepared and plated using the pour plate technique in triplicate (Vandeweyer et al., 2017). Total viable counts were assessed after aerobic incubation on PCA and NA medium for 72 h at 30 °C.

2.7. Isolation of plastic-degrading microorganisms

To isolate plastic-degrading microorganisms two methods were used. In the first step the clear zone test was applied. A cell suspension (100 μl) was spread across FMM plates containing 0.1% emulsified PS, PBSA, PBS or PCL as a carbon source. The incubation of plates was at 28 °C for 72 h. Single colonies appearing at the center of the clarified zone on the surface of medium containing emulsified plastic were selected and isolated as plastic-degrading strains. In order to obtain pure cultures they were isolated and sub-cultured repeatedly and stored in 70% glycerol at −80 °C.

To confirm biodegradable activity of isolated strains, the pure cultures were tested in the scratch test. The biomass was inoculated as a scratch on agar plates containing plastic emulsions as a carbon source. After incubation (28 °C, 5–7 days), the clearance zones were observed. The colonies forming clear zones were selected for further analysis.

2.8. Sample preparation, DNA extraction and identification of isolated bacteria

The bacteria with the ability to degrade plastic isolated from larvae of *T. molitor* were identified by 16S rRNA sequence analysis. gDNA was isolated from pure cultures of bacteria using the GeneMATRIX Bacterial & Yeast Genomic DNA Purification Kit (EURx, Poland). The bacterial 16S region was amplified by 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) as primers, which has been described previously (Devereux and Wilkinson, 2004). The sequencing result was compared with 16S ribosomal RNA sequences using the Basic Local Alignment Search Tool (BLAST) in the GenBank National Center for Biotechnology Information (NCBI) database. The 16Sr RNA gene sequences of the isolated bacteria were submitted to GenBank under the accession numbers MF959938, MF959939 and MF959937 respectively. The strains were deposited in the Polish Collection of Microorganisms (WFCC, No. 106), Wroclaw, Poland under numbers: *S. marcescens* PCM3034, *P. aeruginosa* PCM3035, *K. oxytoca* PCM3036.

2.9. Metagenetic analysis

To perform the metagenetic analysis, next-generation sequencing was used. Metagenomic analysis of the 16S rRNA gene subunit was carried out on the basis of the V3-V4 hypervariable region of prokaryotic 16S rRNA. The primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) were used to amplify the selected region and to prepare the library (Takahashi et al., 2014; Thijs et al., 2017). The experiment was done using a Q5 Hotstar High-Fidelity DNA Polymerase (NEBNext). The conditions of the reaction were provided in accordance with the manufacturer's recommendations. The sequencing was done on the MiSeq sequencer, with paired-end (PE) technology, 2 × 250 nt, using a set of MiSeq Reagent Kit v2 reagents, according to the manufacturer's instructions (Illumina). Automatic data analysis was carried out on the MiSeq device using the MISeq Reporter software (MSR) v2.6, 16S Metagenomics protocol. The three stages of analysis was performed: automatic demultiplexing of samples, generating fastq files containing raw reads and classification of paired-out readings in individual taxonomic categories. The classification of readings to the species level based on the Greengenes v13_5 reference sequences database, was provided by 16S Metagenomics Protocol (modified by Illumina). Preparation of the reference sequence database included: filtering out sequences with a length of less than 1250 base pairs; filtering out sequences containing more than 50 degenerate pairs and filtering of incomplete classified sequences. The sequence data have been deposited in NCBI; the BioProject accession number is PRJNA550106 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA550106).

2.10. Biochemical analyses

For biochemical analyses we prepared a homogenate from larvae after finishing the experiment, we use a pestle and mortar and we introduced an ice-cold 0.1M phosphate buffer pH 7.4, 1 mM EDTA (1:10 w/v). Total sugar was assessed with anthrone reagent according to methodology of Van Handel (Van Handel, 1985). For total lipid content we applied chloroform: methanol (1:2 v/v) and then homogenate was treated with vanillin reagent (Van Handel, 1985). The left homogenate was then centrifuged at 11 000×*g* (5 min) and we used Bradford method for determining the soluble protein content (Bradford, 1976).

2.11. Statistics

We applied Statistica software package for statistics. For the calculation of significant differences between the beginning of the experiment and the end of the experiment for mass loss among studied PS species we used the paired samples *t*-test. The results were analyzed for statistical significance of the differences in total content of proteins, sugars and lipids for mealworms fed with different PS species with one-way ANOVA and post hoc Tukey HSD test; $p < 0.05$.

3. Results

3.1. Material characteristics, mass changes in mealworms and PS species

We applied four tests to assess the PS species used. Hardness was studied among four materials with the highest results obtained for polystyrene used for packing purposes (PSp) and the lowest for raw (PS) and expanded polystyrene (EPS) (Fig. 1A). The gloss of

studied materials was highest for PS and PSr in contrast to PSp and EPS (Fig. 1B). Melt flow ratio (MFR) characterizes the possible differences in molecular structure of materials (Fig. 1C). The lowest value of MFR was obtained for PSp, whereas PSr and EPS revealed higher values (from 2 to 3.5 times); for PSr it is probably the result of the recycling process, which influenced the value of MFR. The contact angles so called wetting angle assess the wettability of a solid by a liquid (Fig. 1D). The highest values of contact angle were also obtained for PSr and EPS materials.

In contrast to the other studies (Yang et al., 2014, 2015a; Yang et al., 2015b), we did not observe that larvae of mealworms can eat and degrade polystyrene. Mass changes of larvae of *T. molitor* were recorded after 21 days of the experiment (Table 1) and in all variants a loss of mass was observed with the exception of the control (mealworms fed oatmeal) and imago variants. The highest mass loss was recorded for larvae fed with PS (raw polystyrene), which suggests that in such form the material is not available for larvae. For all types of materials, no mass increase was observed; on the contrary, Table 1 clearly shows that mealworms lost a substantial fraction (up to 20%) of their body mass if fed only on various types of polystyrene. Additionally, we studied mass loss of used materials (Supplementary Data Table S1). The largest mass loss was observed for the PSp and EPS (16.07% and 19.3% respectively at the end of the experiment), which is consistent with the results obtained for mass changes of *T. molitor* (the lowest mass loss was recorded for larvae fed with PSp and EPS). Significant differences were observed between the 1st day and 21st day (the end of the experiment) for mass loss among studied PS species ($p = 0.001$, *t*-test). Our results show clearly that all these polymers do not support development of the larvae.

Table 1
Mass changes of mealworms caused by waste eating and starvation larvae (g). "-" mass decrease.

Variant	Mass changes of *T. molitor* after 21 days (%)
Control	+36 ± 3
PS	−20 ± 4
PSr	−15 ± 2
PSp	−12 ± 6
EPS	−10 ± 2
Starved larvae	−14 ± 3
Imago	+4 ± 1

3.2. Biochemical analyses

The total protein level in larvae fed with various PS species at 21st day of experiment was almost constant compared to the content of protein in control *T.molitor* fed with oatmeal. For adults the content of proteins was higher (Supplementary Material Table S2). The lowest value was recorded for starved larvae and also for mealworms fed with PS and PSp. The total sugar content was lower of 10.69% in comparison to control. The lowest value was recorded for starved mealworms and mealworms fed with PS. The total lipid content decreased by up to 43.60% in relation to control. The lowest value was obtained for starved individuals, then PS and PSr. The analysis of variance (one-way ANOVA) showed significant differences among three studied metabolites according to feeding type ($p < 0.0001$). The results show the PS species with statistically significant differences for content of protein, sugar and fat (Tukey

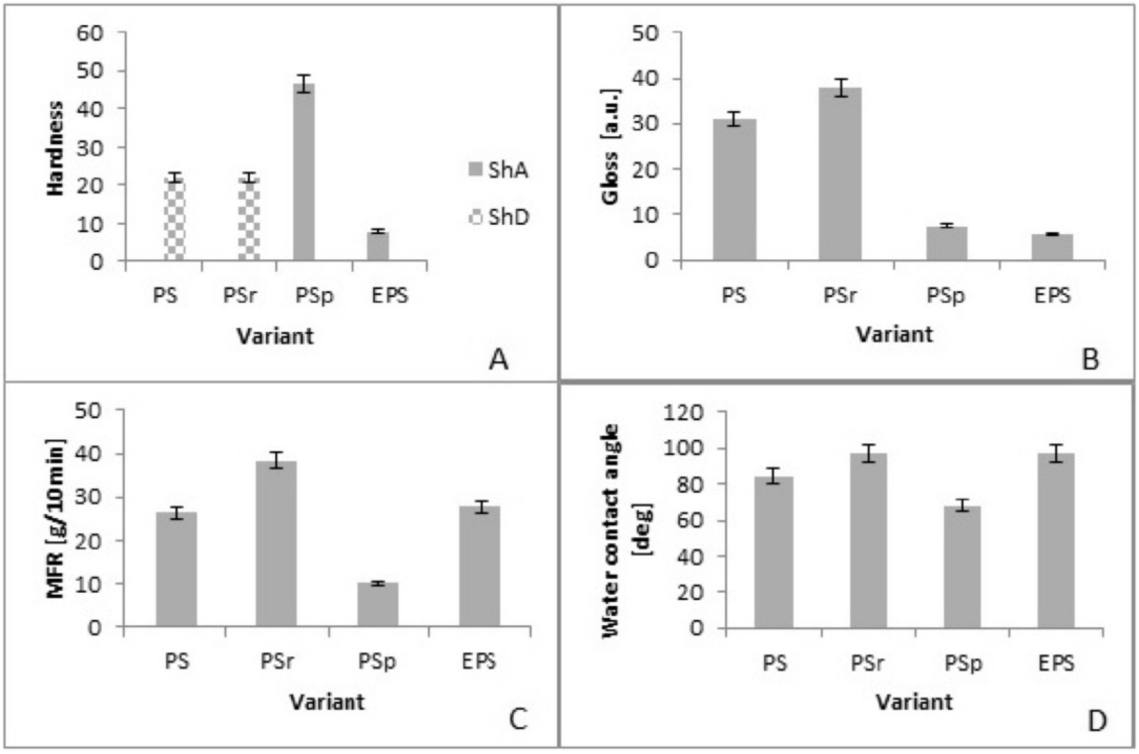

Fig. 1. A. Shore hardness of mealworms fed materials (PS, PSr, PSp, EPS) ± SD. Shore D (for PS and PSr) and Shore A scales (for PSp and EPS samples); B. Gloss of studied materials ± SD; **C.** Melt flow ratio (MFR)± SD of PS species; **D.** Water contact angle ± SD of PS species.

HSD post-hoc test). Significant differences were found for all variants with a few exceptions (Supplementary Material Table S3).

3.3. Comparison of the gut microbiome between individuals fed with different polystyrene types

First, the total number of colony forming units (CFUs) on the plates was determined by counting each colony in a tenfold dilution series. Colonies were counted after plating out the stock seeds of larva guts and incubating them for 72 h at 28 °C. Fig. 2 presents the results of this experiment. The source of the stock seeds was crushed guts dissected from mealworms, which were differently fed. The highest number of bacteria was observed for larvae fed with PSp (44.325×10^6 CFU). Due to its expanded properties and lightness, PSp was the material consumed fastest by mealworms. Surprisingly, larvae fed with commercially available PS (EPS) showed a low number of bacteria in their guts (14.3). The highest abundance of the bacteria was observed for the control.

The next step in this study was NGS analysis to verify the dissimilarities in bacterial community composition between individuals fed with different sorts of PS. During this experiment all variants, i.e. starved larvae, larvae fed with PSp, PS or PSr, control and imagines, were tested. First, the reference sequence database was prepared. Sequences with a length of less than 1250 base pairs, sequences containing more than 50 degenerate pairs and incomplete classified sequences were filtered out. The sequencing statistics shows that at least 91% of all reads passed quality filtering (Supplementary Material Table S4).

Through NGS it was proved that almost all, i.e. 99.9% (±0.000849), of reads were classified as belonging to the bacteria kingdom. Taxa assignments were performed and showed that the most abundant microorganisms in the *T. molitor* microbiomes are members of the phyla *Proteobacteria* and *Firmicutes* (68.68% ± 10.44 and 30.97% ± 10.50 respectively). In the case of imagines, the fluctuations were significantly different. The occurrence of *Proteobacteria* reads was represented at the level 99.39% whereas *Firmicutes* was represented by 0.37%. At the class level, it was found that bacteria belonging to *Gammaproteobacteria*, *Bacilli*, *Clostridia*, *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria* and *Flavobacteria* were the most common in all samples. The relationship between bacterial classes and the tested samples is presented as a circle diagram in Fig. 3.

Fig. 4 shows a classification at the species level of each sample. In the presented pie charts the top (among all) species-level taxonomic categories are shown. Data show that in almost all investigated larval types the predominant bacterial species was *Enterobacter hormaechei* (32.65% of the whole metagenome in control larvae; 26.21% in larvae fed with EPS; 30.37% in PSr; 23.47% in PSp; and 25.64% in starved larvae). In the control sample of larvae *E. hormaechei* was strongly supported by *Lactococcus garvieae* (30.04%). This bacterium was the most abundant in guts of PSp larvae (37.71%). Moreover, in this sample, besides *E. hormaechei* and *L. garvieae*, a high concentration of *Enterobacter aerogenes* was found (16.20%). Taking into account the fact that PSp was the easiest material for larvae to eat and the abundance of bacteria in the gut was at the highest level among all types of PS, the presence of these bacteria may be associated with biodegradation abilities and they may take part in digestion of PS. In the case of EPS and PSr, respectively *Enterococcus avium* (25.05%) and *Lactococcus lactis* (13.67%) were also observed as significant components of microbiota. An interesting observation is the presence of *Klebsiella oxytoca* at a high level in starved larvae (12.15%). Its presence was also confirmed in other variants: PSp – 0.82%; PSr – 0.25%; EPS – 0.43% and I – 0.69%. It was not found in the control sample. It should be noted that in the samples the bacterial abundance showed a wide variety, but the number of reads was definitely at a lower level. The total differences in bacterial community composition at the all taxonomic levels between samples with different ways of feeding and between larvae and imagines are visualized through the stacked column charts (Supplementary Material Fig. S1 and Fig. S2, respectively).

Focusing on the taxonomic level of imagines and larvae, it can be noted that the larva bacterial community was differently diverse than the imago bacterial community. First of all, the presence of *E. hormaechei* was not observed in imagines. The vast majority of imagines were *L. lactis* (41.42%) and *L. garvieae* (40.88%), which also was found in larvae.

3.4. Identification of plastic-degrading microorganisms

The next step in this study was isolation and identification of the plastic-degrading bacteria. For this reason gut cell suspensions

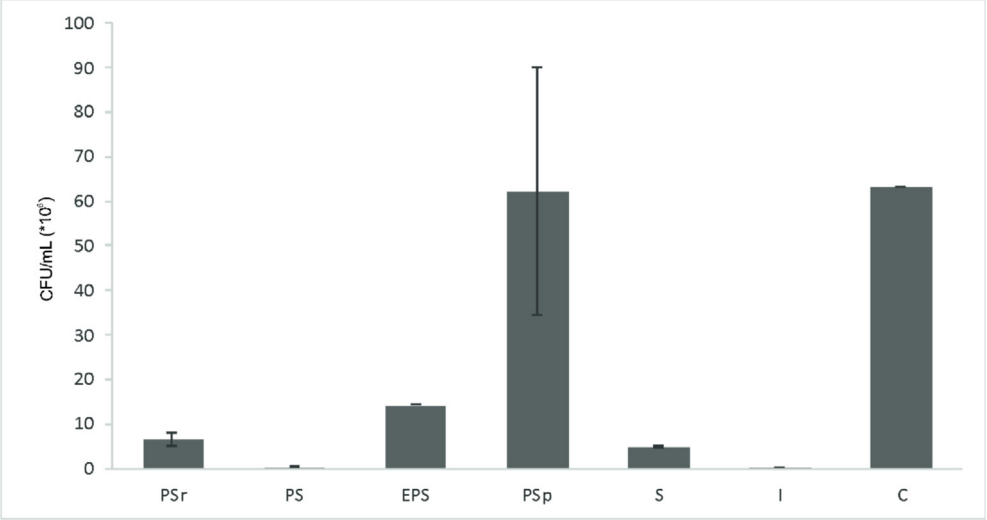

Fig. 2. Bacterial counts for the various breeding groups of mealworm larvae and imagines of *T. molitor*. Individuals were fed with: PSr – larvae fed with processed polystyrene; PS – larvae fed with raw polystyrene; EPS – larvae fed with expanded polystyrene; PSp – larvae fed with material commercially available for parcels; S – starved larvae, I – imagines; C – larvae fed with oatmeal *ad libitum* as a control. The bars show standard deviation.

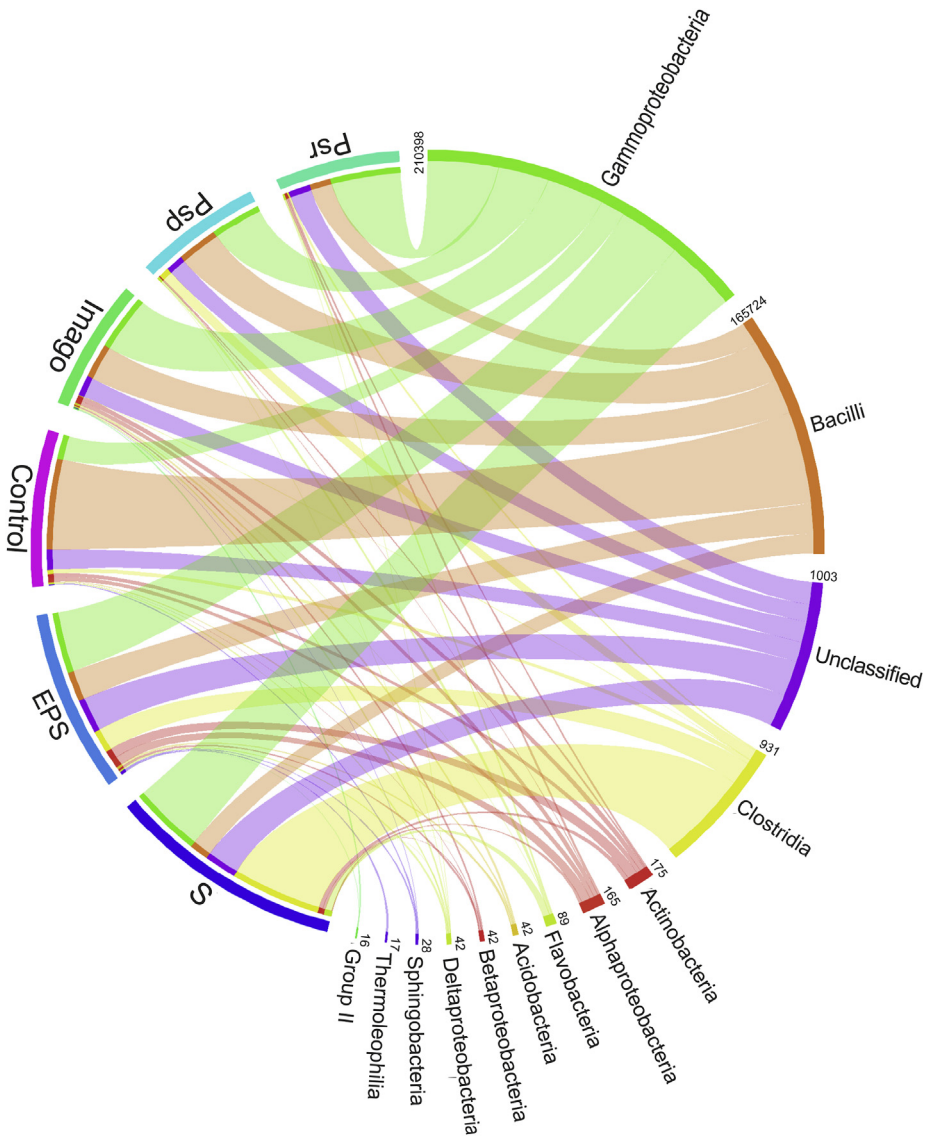

Fig. 3. Circular visualization of occurrence and abundance in class-level taxonomic categories in larvae and imagines. Thickness of the ribbon refers to number of reads of each bacterial class assigned to imagines or larvae in case of different ways of breeding (PSr, PSp, Imagines, Control, EPS and S) obtained by NGS. The ring summarizes the total number of reads assigned to each bacterial class whereas the relative abundance of reads present in the larvae and imagines is represented in the innermost rings. The majority of the microbiome is shared by all insects. Identification to the class level was performed using the Greengenes v13_5 reference sequence database, modified by Illumina. Data were parsed with Circos table viewer (Krzywinski et al., 2009).

were prepared and spread onto FMM plates containing 0.1% emulsified PS, PBSA, PBS or PCL as a sole carbon source. After incubation clear zones could be observed around bacterial colonies. We isolated 11 bacterial strains that were tested in scratch assays to confirm their biodegradability. Next, single colonies were applied onto FMM agar plates and incubated for 48 h at 28 °C. After this period we isolated three different strains that showed degrading ability on the plates with bioplastics as a sole carbon source. These strains were selected for further tests and their capability of bioplastic degradation was confirmed during well diffusion assays (Fig. 5). Unfortunately, for pure culture the formation of a clear zone was not observed with PS as a sole carbon source (data not shown). The next step was identification of the selected bacterial strains. The strains were identified as *Serratia marcescens*, *Klebsiella oxytoca* and *Pseudomonas aeruginosa*. As mentioned above, *K. oxytoca* was found in all samples except for the control.

4. Discussion

In 2017 the world plastic production extended to almost 350 million tonnes (Aisbl, 2018) of which approximately 50% were used for single-use disposable applications. As a result of such huge production and usage, plastics frequently end up and accumulate in landfill and ocean debris, causing environmental pollution (Ho et al., 2018; Hopewell et al., 2009). As a result, conventional ways to tackle plastic pollution such as recycling or energy recovery are not sufficient anymore. Therefore, many other methods are developing now. Among them, one solution might be the application of microorganisms' biodegradable activity to remove plastics (Zheng et al., 2005). The biodegradable activity of microorganisms has been proved many times. However, the current research still should be focused on searching for new microorganisms with the ability to break down not only biodegradable plastic, but also conventional

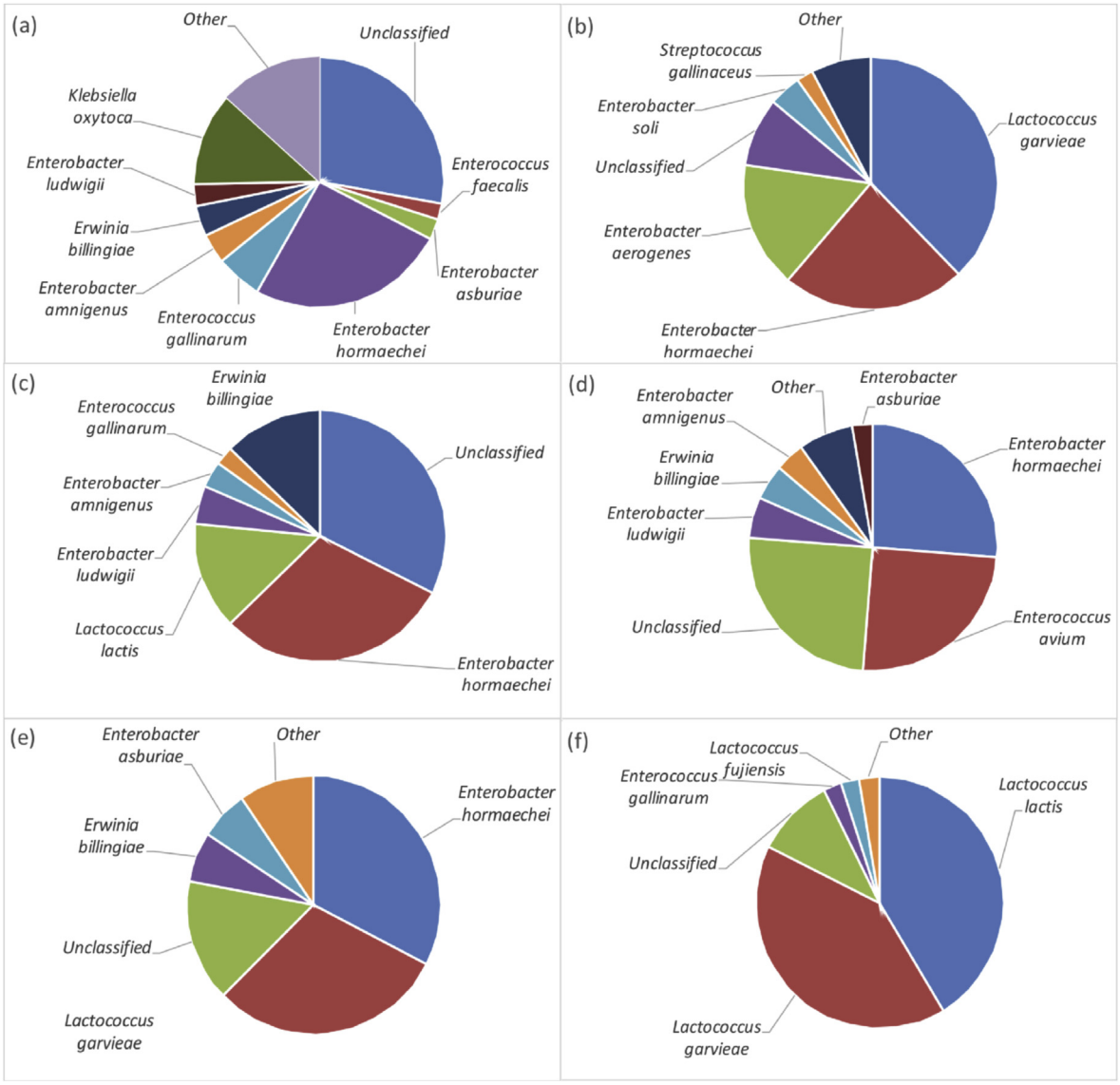

Fig. 4. Top species classification results in different samples. The pie charts show the top (among all) species-level taxonomic categories identified in each sample depending on the way of breeding as follows: (a) starved larvae (total species identified: 185); (b) PSp (total species identified: 153); (c) PSr (total species identified: 98); (d) EPS (total species identified: 237); (e) control (total species identified: 149); (f) imagines (total species identified: 101). The "Other" category is the sum of all classifications with less than 2.0% abundance. The "Unclassified" category refers to bacteria unclassified at the species level. Identification to the species level was performed using the Greengenes v13_5 reference sequence database, modified by Illumina.

plastic, such as PS.

The first stage of our research was to investigate the properties of PS materials that were used in this study (PS, PSr, PSp and EPS) to assess their availability to mealworms. Unfortunately, we did not find the most preferable material for consumption, although the mass loss for PSp and EPS was the highest during the experiment (16% and 19% respectively; Supplementary Material Table S1). The differences in mass of both materials, PSp and EPS, ranging from 16 to 19%, are not significant and could be interpreted as within the range of error. However, susceptibility of the material surface to larval biting could be associated with the particular properties of studied materials (Fig. 1A), as hardness increased as follows: EPS < PSp < PS < PSr (with PSr and PS being measured on the Shore D scale). In recent study, the mass loss of the PS material used for mealworm feeding was 12.2% (Przemieniecki et al., 2019). The results of gloss measurement (Fig. 1B) are also compatible with Shore hardness measurements. In general, the more glossy the surface is, the less susceptible it is for larval biting. The surfaces of PSp and EPS were less glossy in comparison to raw PS and PSr, which suggests their greater availability for biting. Other parameters measured such as MFR and contact angle (Fig. 1C and D) are characterized by the highest values for PSr material and the lowest for PSp (MFR and contact angle). The lowest MFR may indicate that mean polymer chain length was at its lowest value; thus that system could be the most easily chewed and bitten. Moreover, the lowest contact angle (for PSp) suggests that this surface had the most hydrophilic character and thus was most prone. On the other hand, the highest MFR indicates the longest polymer chains, which could prevent it from larval chewing, although we obtained quite high values for EPS. The contact angle shown in Fig. 1D (the highest for PSr) indicates that interaction between water suspension/solution and polymer surface is the lowest for the discussed systems.

To summarize, in this study the obtained data suggest that mealworms can bite and chew studied polymers with various

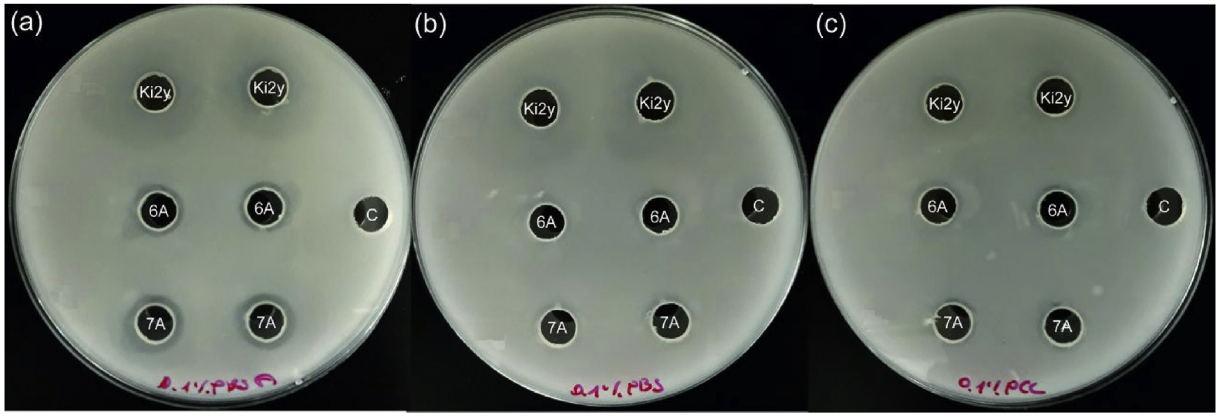

Fig. 5. Biodegradable ability of three isolated strains: *Pseudomonas aeruginosa* (Ki2y), *Serratia marcescens* (6A) *and Klebsiella oxytoca* (9A). To each well 100 μl of bacterial suspension was applied, plates were incubated at 28 °C for 48 h. The control (C) was sterile water. (a) – clear zones on FMM medium containing 0.1% PBSA; (b) – clear zones on FMM medium containing 0.1% PBS; (c) – clear zones on FMM medium containing 0.1% PCL.

effectiveness depending on their properties but there was no strong indication for their utilization (digestion/degradation). In our opinion, the ability to chew and bite polystyrene was developed in these insects as they are warehouse pests and must have the ability to access the grain, which is usually stored in plastic containers.

The results describing the total protein, sugar and fat content depending on food types served to mealworms (PS, PSr, PSp, EPS and starved larvae) are consistent with the mass loss of larvae fed with various food species and with materials' characteristics (Supplementary Material). In all studied variants changes were recorded in the total lipid content; it declined significantly in mealworms fed with PS, PSr, and to a lesser extent for EPS and PSp, suggesting that mealworms are starving as Ps species were nearly not available for consumption. The most noticeable changes during starvation always happen in fat level as the oxidation of fatty acids stored in the form of triglyceride is a main and the most important energy source in insects during malnutrition (Renault et al., 2002). In the present study, we also recorded the decreased level of carbohydrates in mealworms fed on all types of polystyrenes, although the greatest changes were observed for raw PS and PSr, which is probably the result of materials' properties. In some studies on insects the level of carbohydrates was little affected during starvation, e.g. studies on starvation depending on the temperature in *Alphitobius diaperinus* (Renault et al., 2002), although other studies found that carbohydrates can be rapidly used and were nearly depleted during food shortage, e.g. in African fruit beetle *Pachnoda sinuata* (Auerswald & Gäde, 2000). Trehalose is the main carbohydrate in the insect body, but glucose is also present in the hemolymph and plays crucial role for maintaining organs in good functioning, particularly the nervous system. Thus the great decrease in carbohydrates may indicate a shift from lipids to carbohydrates, which is probably the case in our experiment.

The protein level was the most stable and resistant to changes and only slightly affected by food type. Significant differences naturally were found between control and PS, control and PSp, control and EPS but also among starved and all other variants (C, PS, PSr, PSp, EPS and I), imagines and all other variants (C, PS, PSr, PSp, EPS and S) (Supplementary Material), suggesting that starvation influenced metabolism of larvae faster than feeding on types of PS, which is probably a result of attempts of eating inedible forms of PS. Hydrolyzed proteins are used as energy for metabolism or converted to glucose during gluconeogenesis; thus during food deprivation a decline of protein level is observed (Renault et al., 2002).

The NGS study showed differences in the gut microbiome. In our study the bacterial community found in mealworms was primarily composed of two phyla, *Proteobacteria* and *Firmicutes*. A low percentage of *Acidobacteria* and *Bacteroides* was also detected. Similar results have been obtained in other research on mealworms fed with PS as well, although in that study a high percentage of *Bacteroides* was detected (Przemieniecki et al., 2019). Unclassified bacteria accounted for 0.21% of the bacterial community. In the previous studies, mostly three dominant phyla were indicated in the bacterial communities: *Proteobacteria*, *Firmicutes* and either *Tenericutes* (Jung et al., 2014; Wang and Y, 2015; Wynants et al., 2017), or *Actinobacteria* (Stoops et al., 2016). Wynants et al., (2017) additionally detected a low abundance of *Acidobacteria* and *Bacteroides*. A similar result was obtained by Garofalo et al., (2017). *Tenericutes*, *Proteobacteria* and *Firmicutes* have been reported to have high abundance and *Bacteroides* and *Actinobacteria* low abundance in mealworm larvae (Garofalo et al., 2017).

Surprisingly, in many studies at the genus level, the most prevalent genus was *Spiroplasma* sp., which is generally regarded as a pathogen or male-killing bacterium in insects, (Brandon et al., 2018; Jung et al., 2014; Wang and Y, 2015; Wynants et al., 2017; Zhao et al., 2005). In our study we did not observe the presence of *Spiroplasma* sp. in the metagenome of mealworms. Nonetheless, at a varied level we noted the presence of *Enterobacter*, *Klebsiella*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Erwinia* and *Paenibacillus*, some of which were found previously in the digestive systems of mealworms. Interestingly, according to the previously published report (Brandon et al., 2018) the most abundant bacterial taxa in mealworm fed PS were *Enterococcus* spp., *Listeria* spp. and *Nitrospira defluvii*. Moreover, Stoops et al. (2016) also detected bacteria belonging to *Propionibacterium* as the most abundant genus and additionally *Streptococcus*, *Haemophilus*, *Staphylococcus*, *Lactobacillus*, *Pseudomonas* and *Clostridium* (Stoops et al., 2016). All of these results show that bacterial community composition may be completely different among samples within the same host. Jung et al. (2014) tested nine individual mealworms and noted that the bacterial communities were not identical across all individuals (Jung et al., 2014). This observation confirms the theory of Cariveau et al. (2014), which suggests that bacterial communities can differ between individuals growing in the same environment (Cariveau et al., 2014). On the other hand, the results might suggest that mealworms from different areas have a part of their microbiome in common.

The evidence of biodegradable activity of microorganisms isolated directly from *T. molitor* guts is poor. Only Yang et al. (2015a, b)

have successfully found bacteria (*Exiguobacterium* sp. YT2) with a capacity for slight devastation of polystyrene films. According to results obtained through NGS, we have not observed existence of *Exiguobacterium* sp. in the metagenome of *T. molitor*. Moreover, in a recent study on *T. molitor* microbiome, Przemieniecki et al. (2019) did not observe *Exiguobacterium* neither. Despite the fact of lack of biodegradation activity against PS, we isolated three bacterial strains able to degrade bioplastic, which was indicated in agar plate tests (Fig. 5). We identified bacterial strains as belonging to the species *Serratia marcescens*, *Klebsiella oxytoca* and *Pseudomonas aeruginosa*. Interestingly, the presence of *K. oxytoca* was detected in all tested samples (except for the control). There is a possibility that this bacterium is engaged in the digestion process in the intestinal system of *T. molitor*. Moreover, *Enterobacter hormaechei* found as a major bacterium in all PS-fed samples can also be considered as a bacterium taking part in plastic digestion. However, whereas *Klebsiella* and *Serratia* genera were found in gut bacterial communities (Osimani et al., 2018), the presence of *E. hormaechei* has not been detected yet in other studies. Only presence of bacteria belonging to the genus *Enterobacter* was confirmed.

The role of the microorganisms residing in *T. molitor* is not fully understood (Jung et al., 2014). It can be assumed that the digestion process occurring in the intestine of mealworms is more complex than it seems. Perhaps the role of whole microbiota and synergic relations between bacterial species are important in the aspect of the PS biodegradation process. A recent study showed that *T. molitor* from diverse locations is able to survive on PS and this feature is independent of the geographic origin of the mealworms (Yang et al., 2018). On the other hand, in another study, differences between mealworms from diverse locations were found (Wu et al., 2019). That study indicated that *T. molitor* from different regions presents different metabolic effects. These results are in agreement with our findings. Interestingly, in all studies it was found that the differences in microbiota depend on the supplied plastic.

The obligate symbionts very often support their hosts with vital dietary factors that are not present in the natural diet (Pontes & Dale, 2006). Insects living on nutrient poor diets, where essential compounds such as amino acids are very scant, benefit from symbiotic bacteria due to their rapid ability to adapt to diet changes of the host by altering the population profiles easily and induction of essential enzymes (Genta et al., 2006), which could occur in the case of PS-degrading bacteria and *T. molitor*. Based on this, we assume that mutual relations between the hosts and their microbiota are as important as synergic actions between bacterial species living in the guts. Particularly, molecular phylogenetic research proved that majority of the obligate endocellular symbionts of diverse insects commonly belong to the same bacterial group *Proteobacteria* (Hosokawa et al., 2006), which is detected the most frequently in the gut microbiota as mentioned above. For instance, in gut tissue tsetse flies have two symbiotic microorganisms: the obligate primary-symbiont *Wigglesworthia glossinidia* and the commensal secondary-symbiont *Sodalis glossinidius* (Akman et al., 2002).

5. Conclusions

In this study was found that the microflora of the *T. molitor* gut varies depending on the supplied type of polystyrene and the developmental stage and is strongly related to the type of supplied PS. Next, we isolated bacterial strains from the gut of *T. molitor* and tested their plastic-degrading properties against bioplastics such as PBSA, PBS and PCL. The bacteria belong to species the *Serratia marcescens*, *Klebsiella oxytoca* and *Pseudomonas aeruginosa*. The data suggest that unconventional environments, such as the guts investigated in the present study, may be a good source of microorganisms with efficient biodegradation ability. The more peculiar the environment, the greater is the chance of finding interesting microorganisms with extraordinary abilities. Therefore investigation into plastic-degrading microorganisms should be continued. To clarify the degradation mechanism and community behavior of the gut microbiota in more detail, further research should be conducted.

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Declaration of competing interest

The author(s) declare no competing interests.

CRediT authorship contribution statement

Aneta K. Urbanek: Investigation, Formal analysis, Validation, Writing - original draft. **Justyna Rybak:** Conceptualization, Resources, Validation, Writing - review & editing. **Magdalena Wróbel:** Investigation, Validation, Writing - original draft. **Karol Leluk:** Methodology, Writing - review & editing. **Aleksandra M. Mirończuk:** Conceptualization, Funding acquisition, Writing - review & editing, Supervision.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114281.

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