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Biodegradation of polyethylene and plastic mixtures
in mealworms (larvae of *Tenebrio molitor*) and
effects on the gut microbiome

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**KEYWORDS:** Polyethylene, biodegradation, plastic waste, polystyrene, mealworms, *Tenebrio molitor*
ABSTRACT

Recent studies have demonstrated the ability for polystyrene (PS) degradation within the gut of mealworms (*Tenebrio molitor*). To determine whether plastics may be broadly susceptible to biodegradation within mealworms, we evaluated the fate of polyethylene (PE) and mixtures (PE+PS). We find that PE biodegrades at comparable rates to PS. Mass balances indicate conversion of up 49.0 ± 1.4% of the ingested PE into a putative gas fraction (CO$_2$). The molecular weights (M$_n$) of egested polymer residues decreased by 40.1 ± 8.5% in PE-fed mealworms and by 12.8 ± 3.1% in PS-fed mealworms. NMR and FTIR analyses revealed chemical modifications consistent with degradation and partial oxidation of the polymer. Mixtures likewise degraded. Our results are consistent with a non-specific degradation mechanism. Analysis of the gut microbiome by next-generation sequencing revealed two OTUs (*Citrobacter* sp. and *Kosakonia* sp.) strongly associated with both PE and PS as well as OTUs unique to each plastic. Our results suggest that adaptability of the mealworm gut microbiome enables degradation of chemically dissimilar plastics.

INTRODUCTION

Plastics are a growing concern for both the environment and waste management systems. Global plastic production has tripled in the last 25 years to over 322 million tons in 2015.\(^1\) This nearly exponential growth in production has contributed to waste management challenges including space limitations in landfills, which coupled with low recycling rates has led to mismanagement of plastic waste and increased environmental pollution.\(^1\text{–}^3\) Plastic is of especially great concern in marine environments where its recalcitrance has led to accumulation and harmful effects on wildlife and potentially humans.\(^4\text{–}^5\) To combat this growing problem, there has been a steady increase in research on plastic biodegradation by bacteria and fungi.\(^6\text{–}^8\)
Microbial enrichment and isolation studies have demonstrated that several bacterial isolates are capable of degrading plastics, but rates of degradation vary and are typically low.\textsuperscript{6–8}

Recent work has demonstrated that mealworms (larvae of \textit{Tenebrio molitor}), obtained from various sources across the globe, readily ingest and biodegrade polystyrene (PS) to CO\textsubscript{2} and lower molecular weight compounds within their gut.\textsuperscript{9–11} Antibiotic studies implicated gut bacteria as agents of PS degradation, and \textit{Exugiobacterium} sp. YT2, a bacterium capable of PS degradation was isolated from the gut.\textsuperscript{10} Mealworms were shown to degrade nearly half of the ingested PS within the 12 – 15 hour retention time in the gut, which is higher than the mass loss reported by the isolated \textit{Exugiobacterium} sp. YT2 and other plastic-degrading bacterial isolates.\textsuperscript{9,10,12} A recent report found that co-feeding PS with bran almost doubled the rate of PS degradation.\textsuperscript{11}

Mealworms are omnivorous and researchers hypothesize that their gut bacteria play an important role in their ability to adapt to different foods.\textsuperscript{13} Bacterial concentrations range from \(10^5\) to \(10^6\) colony forming units per gut.\textsuperscript{10,13} On a standard diet (e.g., bran), the anterior gut is dominated by facultative anaerobes of the genera \textit{Lactococcus} and \textit{Pantoea} and by genera within the family Bacillaceae, while the posterior gut is more diverse, featuring anaerobes from the genera \textit{Spiroplasma}, \textit{Clostridium}, and \textit{Enterobacter}.\textsuperscript{13}

To determine whether plastics may be broadly susceptible to biodegradation within mealworms, we evaluated the fate of polyethylene (PE) within the gut. Assessing the degradation of PE is of interest because PE has a markedly different chemical structure than PS; PE lacks a benzene ring in the repeating monomer unit, which could impact the resulting degradation. The chemical structure of polyethylene represents the simplest homogenous carbon-to-carbon backbone.\textsuperscript{14} Thus, the ability to degrade PE would suggest that the mealworm gut microbiome is
capable of degrading other plastics with a similar chemical motif (e.g., polyvinyl chloride or polypropylene). In addition, the global production of polyethylene (PE) is approximately four times that of PS, and PE plastics are among the most common plastic pollutants.\(^{14,15}\)

There have been recent reports of PE degradation within the gut of other omnivorous insect larvae (Indian meal moths, wax moths), which could suggest that the insect gut broadly enables the degradation of recalcitrant plastics; however, evidence for degradation is preliminary and warrants further study and confirmation.\(^{16,17}\) None these studies reported the ability to degrade more than one type of plastic or plastic mixtures. Additionally, few previously identified plastic-degrading systems, including isolated microorganisms, are capable of degrading multiple types of plastic, suggesting that the plastic degradation is typically plastic-specific.\(^{6–8}\)

In this study, we investigate the biodegradation of polyethylene (PE) and mixed plastics (PE and PS) in a previously untested strain of \(T.\ molitor\). We find that PE degrades at rates comparable to those of PS-fed larvae.\(^{9,11}\) Moreover, mixed plastics (PE and PS) are consumed and degraded, suggesting that degradation is non-specific. Next-generation sequencing indicates that this degradation is associated with changes in the gut microbiome.

**MATERIALS AND METHODS**

**Mealworm survival and plastic consumption.** Mealworms, larvae of \(T.\ molitor\) Linnaeus, (average weight 75-85 mg/worm) were purchased online from Rainbow Mealworms (Compton, CA) and shipped overnight to the laboratories at Stanford University. Prior to arrival, the mealworms were fed bran; after arrival, they were subject to a 48-hour starvation period before initiating experimental diets. Natural wheat bran was purchased from Exotic Nutrition (Newport News, VA).
Six experimental diets were compared: PE, PE + bran (1:1 [w/w]), PS, PS + bran (1:1 [w/w]), PE + PS (1:1 [w/w]), and bran (control diet). To assess mealworm survival rate and plastic mass loss, 120 randomly selected mealworms were placed in a food grade polypropylene container (volume 475 mL) along with 1.80 g of plastic cut into 2 – 3 cm cubes (PE, PS, or 0.90 g PE and 0.90 g PS). Bran-fed containers (PE + bran, PS + bran) initially received 1.80 g bran plus plastic spread throughout the container. Additional bran was added every 3 days to maintain a 1:1 ratio [w/w] of plastic to bran within each container. Bran-fed controls initially received 1.80 g of bran and 1.80 g of additional bran every 3 days. All tests were carried out in duplicate. Containers were stored in incubators maintained at 25 °C and 70% humidity.9,11

Mealworm survival was evaluated approximately every 3 days for 32-days by counting dead mealworms, which were then removed. Once a week, the mealworms were cleaned with a stream of air to remove any residual plastic fragments and transferred to a clean container to collect frass (excrement) for analysis. After 12 hours, the mealworms were returned to their original container, and frass samples were weighed and stored at -20 °C.

To obtain sufficient frass for characterization, 1000 mealworms (from the same order) were raised in larger “bulk-fed” food grade polypropylene containers (volume 780 mL) on the six diets described above. These containers followed the same bran-supplementation and frass-collection schedule as described above. Bulk-fed containers were also stored in incubators maintained at 25 °C and 70% humidity. Frass samples from the end of the 32-day experiment are used in the analysis below.

**Plastic test materials.** To assess the degradation of commercially available plastic products, low-density polyethylene foam, 1.3 cm thickness, was purchased online from the Foam Factory (Macomb, MI). The polystyrene foam, 5.1 cm thickness, used was an expanded polystyrene
insulation material from Carlisle Construction Materials (Puyallup, WA). Plastic foam blocks were cut into irregular 2 – 3 cm cubes and cleaned with a stream of air to remove any residues prior to being placed in the appropriate container. The molecular weight and density of the control materials are shown in Table 1. The PE foam contained no chemical additives. The PS foam contained less than 1% 1,2,5,6,9,10-hexabromocyclododecane (a common flame retardant in PS materials).

**Characterization of plastic degradation within egested frass.** To characterize depolymerized polymer in the frass, the molecular weight (number-averaged $[M_n]$ and weight-averaged $[M_w]$ molecular weight) of the polymer was quantified by high-temperature gel permeation chromatography (HT-GPC) using previously established methods. Frass samples (50 mg) were gently crushed in a mortar and pestle prior to a 2-hour extraction in 2 mL solvent aliquots with gentle heating (placed on a hot plate on the lowest setting). For PE samples, the solvent was dichloromethane ($\geq 99.9\%$, Thermo Fisher Scientific Inc., Pittsburg, PA); for PS samples, the solvent was tetrahydrofuran ($\geq 99.9\%$, Thermo Fisher Scientific Inc., Pittsburg, PA) (SI M1, Fig. S6). After 2 hours, the solution was filtered using a 0.22 µm PVDF filter (Thermo Fisher Scientific Inc., Pittsburg, PA) and transferred into a clean glass vial. The residual polymer in the filtered solution was concentrated by rotary evaporation, and the residue (“residual polymer”) was weighed to determine the extractable fraction (i.e., the fraction of the frass weight recovered). Residual polymer was dissolved in 1,2,4-trichlorobenzene ($\geq 99\%$, Alfa Aesar, Haverhill, MA) to obtain a final concentration of approximately 5 mg/mL. Triplicate analyses for each sample were run at 180 °C with a 100 µL injection volume with an eluent (1,2,4-trichlorobenzene) flow rate of 1.0 mL/min (EcoSEC High Temperature GPC System, Tosoh Biosciences).
Proton nuclear magnetic resonance (\(^1\)H-NMR) analysis was conducted to characterize degradation in the egested frass. The control plastics and residual polymers from mealworms fed each diet were analyzed. Trace residue extracted from the frass of bran-fed mealworms was used as a control. Before conducting the liquid-state \(^1\)H-NMR analysis, frass samples (50 mg) were extracted as described above. The residual polymer was re-suspended in chloroform-D (\(\geq 99.8\%\), Cambridge Isotope Laboratories, Inc., Tewksbury, MA). Proton-NMR spectra were obtained at 55 \(^\circ\)C on a Varian Inova 500-MHz NMR spectrometer (Agilent Technologies, Inc., Santa Clara, CA). The \(^1\)H-spectra [32 scans, delay time (d1) = 0.0 s] were referenced to the residual deuterated-chloroform peak [7.26 ppm]. Spectra were analyzed using MestReNova software (version 10.0.2), values are reported in parts per million (ppm).

Additional characterization of the residual polymer was obtained using Fourier Transform Infrared Spectroscopy (FTIR) on a Nicolet iS50 FTIR Spectrometer (Thermo Fisher Scientific, Inc., Pittsburg, PA). Spectra were recorded from the residual polymers extracted from the frass ground to a homogenous powder in absorbance mode and transformed into transmittance for graphing. Spectra were recorded in the range of 4000-500 cm\(^{-1}\) with a minimum of 16 scans with a spectral resolution of 0.482 cm\(^{-1}\). Peaks were identified using OMNIC software (Thermo Fisher Scientific Inc., Pittsburg, PA). Proton-NMR and FTIR analysis were run in duplicate for each diet on residual polymer collected from the bulk-fed containers.

**Microbial community analysis.** At the end of the 32-day experiment, the gut content of each sample (four mealworms from the same container pooled to eliminate individual variability) was harvested and washed four times by vortexing the guts with 100 \(\mu\)l of DNA extraction buffer (0.1 M NaH\(_2\)PO\(_4\), 0.1 M Na\(_2\)HPO\(_4\), 0.1 M EDTA, 0.1 M Tris-HCl, 1.5 M NaCl, 1% CTAB). Gut walls were removed, and DNA was extracted using the MoBio PowerLyzer PowerSoil protocol
with a 25:24:1 phenol/chloroform/isoamyl alcohol modification to improve yield. Phasing amplicon sequencing was used to sequence the V4 region of 16S rRNA gene (SI M2). Library of the 16S rRNA gene amplicon sequencing were constructed using MiSeq reagent kit (Illumina, San Diego, CA) and DNA was sequenced using an Illumina MiSeq platform.

Sequencing data were processed to combine pair-end reads and filter out poorly overlapped and unqualified sequences using the Amplicon Sequencing Analysis Pipeline (ASAP version 1.3). The sequences (2 x 251 bp) were subjected to quality check with FastQC (version 0.11.5). Pair-end sequences were merged based on the 3' overlap using PEAR (version 0.9.10) with a quality score cutoff of 20 and minimum overlap length of 40 bp. Samples were demultiplexed using split_libraries_fastq.py from the QIIME package (version 1.9.1) based on the barcodes (maximum barcode error of 0 and a trimming quality score cutoff of 20). Primer sequences were trimmed. Dereplication was performed using USEARCH (version 9.2.64) with the command fastx_uniques (with -sizeout for sequence abundance output). Operational Taxonomic Units (OTUs) were clustered using UPARSE (command -cluster_otus of USEARCH) with OTU identity threshold of 97% and singletons and chimeric sequences were removed. Representative sequences of the OTUs were classified using RDP Classifier (training set 16, June 2016) with confidence cutoff of 0.8. Diversity was evaluated using the R package “vegan.” Differential abundance analysis was conducted using the Bioconductor package DESeq2 in QIIME with the Benjamini-Hochberg (BH) correction for multiple testing. Due to sample limitations there was an uneven number of replicates for each diet, with more replicates available for the PE-fed diets (Table S1).

**Statistical analysis.** Statistical analyses were performed in Prism (version 7.0a). To assess differences in survival, plastic consumption, changes in molecular weight, and microbial
diversity ANOVAs were performed, followed by pairwise comparisons using Student’s t-test with Tukey’s correction to assess differences between diets. All p-values are adjusted p-values and all error values are average ± standard deviation.

RESULTS AND DISCUSSION

**PE consumption and effects on survival.** At the end of the 32-day experiment, the survival ratio (SR) of the mealworms fed PE was 98.3% ± 0.0%, a value that was not significantly different (p = 0.92) from that of the bran fed controls (96.3% ± 4.1%) (Fig. 1a). There was also no significant difference (p = 0.65) in SR of mealworms fed PE alone and mealworms fed PE + bran (95.0% ± 1.2%) (Fig. 1a). The SR of PS-fed mealworms (90.8% ± 2.4%) and PS + bran fed mealworms (91.3% ± 1.8%) were similar to values previously reported and were also not significantly different from those of mealworms fed PE (PS: p = 0.06, PS + bran: p = 0.08) PE + bran (PS: p = 0.44, PS + bran: p = 0.54), or the bran-fed controls (PS: p = 0.21, PS + bran: p = 0.27) (Fig. 1a).

Consumption of PE and PS increased throughout the experiment (Fig. 1b). From the initial 1.80 g PE, the total mass loss at the end of the experiment was 0.87 ± 0.0 g by mealworms fed PE (Fig. 1c, d). For mealworms fed PS, the total PS mass loss was 0.57 ± 0.12 g (Fig. 1c, d). For both PE- and PS-fed mealworms, the mass loss was significantly greater when the mealworms received bran as a co-feed. For PE + bran, the mass loss was 1.10 ± 0.12 g and for PS + bran, the mass loss was 0.98 ± 0.11 g (Fig. 1c, d). Specific rates of plastic consumption (mg plastic consumed per 100 worms per day) followed the same pattern (Fig. 1d). The increase in specific consumption when co-fed with bran supports previous findings.\textsuperscript{11}
Evidence for depolymerization and biodegradation of PE. Residual polymer extracted from the frass of the bulk-fed containers was used to assess biodegradation and depolymerization within the mealworm gut. The egested frass contains an extractable fraction, consisting of non-degraded and partially degraded polymer, and a non-extractable fraction (other biological waste not recovered in the extraction process).\textsuperscript{11} A decrease in the extractable fraction (“residual polymer”) suggests that more of the ingested plastic is being completely degraded (mineralized) and/or is being incorporated into mealworm biomass.\textsuperscript{11} The extractable portion of the frass from mealworms fed PE or PS decreased over the course of the 32-day experiment (Fig. 2b).

Depolymerization of PE and PS was characterized using HT-GPC on the residual polymers. HT-GPC analysis of the residual polymers from mealworms fed PE and PE + bran showed a significant decrease in weight-averaged ($M_w$) and number-averaged ($M_n$) molecular weight compared to the control PE (Fig. 2a). The residual polymer from PE-fed mealworms showed an average reduction in $M_w$ of 61.3 ± 5.0% and reduction in $M_n$ of 40.1 ± 8.5% (Fig. 2a, Table 1). The residual polymer from mealworms fed PE + bran showed an average reduction in $M_w$ of 51.8 ± 9.3% and reduction in $M_n$ of 47.6 ± 8.5% (Fig. 2a, Table 1), indicating significant depolymerization of PE occurred within the gut of the mealworms fed PE and PE + bran. HT-GPC analysis of the residual polymer from mealworms fed PS and PS + bran also revealed significant decreases in $M_w$ and $M_n$ compared to the control PS (Fig. S1a, Table 1). The observed depolymerization in mealworms fed PS and PS + bran is consistent with previous observations (Table 1).\textsuperscript{9,11}

Chemical modifications of the residual polymer were examined using $^1$H-NMR analysis. Comparison of the control PE spectra to the spectra of the residual polymer from mealworms fed PE and PE + bran revealed a new peak around 5.3 ppm in a region associated with alkene bonds
(C=C-H) (Fig. 2c, Table S2). This peak was not observed in spectra of control extractions from the frass of bran-fed mealworms, suggesting this peak is not a result of contamination from the frass or the extraction method. Comparison of the control PS spectra to the spectra of the residual polymers from PS and PS + bran fed mealworms revealed new peaks associated with the incorporation of oxygen, previously associated with plastic degradation (Fig. S1c, Table S2).

To further assess the possibility that the newly observed peaks were attributable to contamination by bacterial biomass or secreted proteins, samples of plastic (PE and PS) were separately treated in a suspension of *E. coli* K12 for 48-hours then subject to the same extraction methods (SI M3). The NMR spectra for PE and PS control samples incubated with *E. coli* K12 did not reveal new peaks relative to the control spectra, supporting the conclusion that newly observed peaks in the residual polymers spectra (from PE- and PS-fed mealworms) are evidence of chemical modifications resulting from plastic degradation and are likely not due to contamination (Fig. S2a, b).

Additional evidence of chemical modifications in the residual polymer was obtained by FTIR analysis. FTIR spectra from the residual polymers from mealworms fed PE and PE + bran revealed incorporation of oxygen as indicated by the appearance of peaks associated with C-O stretching (1000-1200 cm\(^{-1}\)) and alcohol groups (R-OH bend, 1300–1450 cm\(^{-1}\); R-OH stretching, 3000–3500 cm\(^{-1}\)) (Fig. 2d, Table S3). These peaks were not observed in the control PE spectra. FTIR spectra for the residual polymers from mealworms fed PS and PS + bran also revealed chemical modifications and the incorporation of oxygen (Fig. S1d, Table S3). Again, to assess whether the new peaks were byproducts of microbial contamination, control plastic (PE and PS) incubated with *E. coli* K12 were also subject to FTIR analysis (SI M3). FTIR spectra of these controls did not reveal any new peaks, supporting the conclusion that the newly observed peaks
resulted from plastic degradation within the mealworm gut and are likely not due to contamination (Fig. S2c, d, Table S3).\textsuperscript{28}

\textbf{Evidence for mineralization via a mass balance.} A mass balance on the plastic-fed mealworms was conducted by measuring the weights of system inputs (plastic and/or bran), outputs (the weight of the frass before extraction and the weight of the extractable fraction), and the weight of accumulated biomass (changes in the weight of surviving mealworms).\textsuperscript{9} The mealworm containers were kept at a constant humidity and all weights were measured as wet-weights to avoid errors due to losses or gains in water vapor. Deviations from a perfect mass balance (e.g., if the outputs ≠ the inputs + changes within the container, “putative gas fraction”) would be due to losses into the gas phase (e.g., mineralization), which were not directly measured.

The putative gas fraction (PGF) increases over the course of the experiment for PE-fed mealworms while the extractable fraction from the frass decreased, both of which suggest more degradation (and mineralization) occurred towards the end of the 32-day experiment (Fig. 2b, Fig. S3a). Mealworms fed plastic alone (PE or PS) gained less biomass weight over the course of the experiment than mealworms co-fed with bran, an observation consistent with previous studies of PS-fed mealworms (Fig. S3).\textsuperscript{11} By mass balance, the PGF was 49.0 ± 1.4% at the end of the experiment for PE-fed mealworms (Fig. S3a). For mealworms fed PE + bran, the PGF was 24.3 ± 2.2% with a higher percent of the ingested mass being incorporated into the mealworm biomass (Fig. S3b). The PGF in PS-fed mealworms was 45.5 ± 2.9%, a value similar to that previously reported for PS mineralization by mealworms (measured via mass balance and \textsuperscript{13}C-carbon tracing) (Fig. S3c).\textsuperscript{9} The PGF of PE- and PS-fed mealworms were not significantly different.
Biodegradation of mixed plastics. When fed a diet of one plastic (PE or PS, with or without bran) there was no significant difference between plastic mass loss by the mealworms based on the plastic (Fig. 1b, c, d). However, when mealworms were co-fed PE and PS (1:1 [w/w]), there was a significantly higher mass loss of PE than PS (Fig. 1c, d). A previous report suggested that differences in plastic consumption rates among different types of PS foams may be due to density, with a higher consumption rate associated with less dense plastics.\textsuperscript{11} In this study, however, the PE plastic had a higher density than the PS plastic, suggesting that other factors may affected the relative rates of mixed plastic consumption. Further work is needed to assess what factors influence differential plastic consumption rates.

The molecular weight distributions of the mixed polymers could not be differentiated via HT-GPC, therefore characterization of depolymerization was not included in this analysis. However, FTIR analysis of the residual polymers revealed chemical modifications and the incorporation of oxygen relative to the control PE and PS spectra, similar to those observed in the residual polymers from mealworms fed PE or PS, which suggests degradation of the mixed polymers occurred within the mealworm gut (Fig. S4, Table S3). This finding offers further evidence that plastic degradation within the mealworm gut is non-specific.

Effects of plastic consumption on the gut microbiome. Next-generation sequencing was used to investigate the effect of plastic diets on microbial community structure (Table S1). Relative abundance analysis revealed the same three majority community members (OTUs) in all diets: \textit{Spiroplasma} sp., \textit{Cronobacter} sp., and \textit{Enterococcus} sp. (Fig. 3a). These three OTUs are common insect gut-associated bacteria and are known members of the \textit{T. molitor} gut microbiome.\textsuperscript{13,29}
We analyzed microbial diversities across all six experimental diets. The alpha diversity of the microbial community, measured via the inverse Simpson index, was not significantly different between the different diets (Fig. 3b). A principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity index revealed clusters associated with different diets, with clear clusters for PE-fed and bran-fed mealworms (Fig. 3c). A multi-response permutation procedure test revealed a significant difference in the microbial communities based on diet (p = 0.001). This suggests that while the majority members of the microbial community do not differ dramatically in PE diets, the composition of the microbial community is distinct from either bran-fed or PS-fed communities.

Differential abundance analysis was used to assess whether particular OTUs were associated with different diets (Fig. 4, Table S4). This analysis revealed two OTUs that were strongly associated (p < 0.05) with both the plastic diets (PE and PS): *Citrobacter* sp. and *Kosakonia* sp. Both OTUs are members of the Enterobacteriaceae, a family known to contain PE-degrading member *Enterobacter absuriae* YT1 isolated from the gut of the larvae of Indian mealmoth.\(^\text{16}\) Both OTUs can utilize oxygen (*Citrobacter* sp. are aerobic, *Kosakonia* sp. are facultative anaerobic), which could be further evidence for their involvement in plastic degradation, as incorporation of oxygen is key in the accelerated biodegradation of both PE and PS, as evident in the analysis of residual polymers and previous work (Fig. 2c, d; Fig. S1c, d).\(^\text{6–8,30}\) Both *Citrobacter* sp. and *Kosakonia* sp. were more abundant (based on relative abundance) in both of the plastic-only diets than the plastic + bran fed diets and were also more abundant than the other OTUs identified via differential abundance analysis (Table S4).

Two OTUs, both minority members of the microbial community, were significantly associated (p < 0.05) with PE-fed microbiomes: *Sebaldella termitidis* and *Brevibacterium* sp. (Fig. 4b, c, d;
Table S4). *Sebaldella termitidis* is phylogenetically isolated within the phylum Fusobacteria, is anaerobic, and is a known inhabitant of the posterior end of the termite gut tract.\(^{31}\) *Brevibacterium* sp. are aerobic bacteria known to be associated with hydrocarbon degradation, including n-alkanes.\(^{32}\) Further work should assess the involvement of *Brevibacterium* sp. in the degradation of polyethylene.

Seven OTUs, all minority members of the microbial community, were significantly associated \((p < 0.05)\) with the PS-fed gut microbiome: *Listeria* sp., *Nitrospira defluvii*, *Pedomicrobium* sp., *Aquihabitans* sp., unclassified Xanthomonadaceae, unclassified Saprospiraceae, and unclassified Burkholeriales (Fig. 4c, d; Table S4). Most of these PS-associated OTUs are aerobic, which is important when considering their possible role in the degradation of polystyrene. The increase in OTUs associated with the PS microbial community could be indicative of a more diverse suite of daughter products created in PS degradation, likely due to the more complex chemical composition of PS and the presence of benzene rings that could degrade into a variety of daughter products. Changes in the PS-microbial community could also be affected by the presence of trace amounts (<1%) of a chemical flame retardant (present in most commercially available PS products). Further research is needed to assess whether and how trace chemicals (especially flame retardants in PS materials) affect the microbial community.

Overall, differential abundance analysis of the gut microbiome revealed several minority OTUs strongly associated with the plastic diets. The gut microbiome, which previous work has shown is necessary for PS degradation, shows changes in response to different plastic diets, further suggesting the importance of the microbial community in the plastic degradation process. While further work is needed to assess the role of individual OTUs in the plastic degradation
pathway, this analysis offers an initial insight into what species might be of interest in future studies.

**Implications.** This work is the first report to demonstrate that PE is depolymerized and undergoes chemical modifications within the mealworm gut. Additionally, we demonstrated for the first time that mixed plastics (PE and PS) undergo biodegradation within the gut. Application of next-generation sequencing to the gut microbiome revealed two OTUs (*Citrobacter* sp. and *Kosakonia* sp.) strongly associated with both PE and PS as well as OTUs unique to each plastic. Our findings suggest that plastic degradation within the mealworm gut is not plastic-specific. This could have implications for future waste management applications.

As the mealworms in this study were previously untested for plastic degradation, their ability to degrade both PE and PS further suggests the ubiquity of plastic degradation among mealworms. Further work is needed to assess whether other recalcitrant plastics that resemble polyethylene (e.g., polyvinyl chloride and polypropylene) degrade in the mealworm gut. Future work should focus on elucidating the mechanisms of degradation within the mealworm to enable future applications.

**ASSOCIATED CONTENT**

**Supporting information.** Additional methods, 6 figures, and 4 tables with details on frass extraction efficiency, sequencing library preparation, plastic characterization controls, and peak information for $^1$H-NMR and FTIR analysis.

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REFERENCES


(13) Wang, Y.; Zhang, Y. Investigation of gut-associated bacteria in tenebrio molitor


Figure 1. Survival rate and plastic consumption by *T. molitor* by diet. (a) Survival rate of mealworms over 32-day experiment. (b) Mass loss in plastic (PE or PS) in the plastic-fed diets over 32-days. (c) The percent mass loss in the plastic by diet at the end of the 32-day experiment. (d) Average specific plastic consumption (mg plastic per 100 mealworms per day) over the 32-day experiment. All values represent mean ± SD, n = 2. Significance (Student’s t-tests, Tukey’s multiple test correction) p < 0.05 indicated by *, p < 0.005 indicated by **, no statistical significance indicated by ns. For mealworms fed PE + PS, the mass loss is displayed separately for each plastic.
Figure 2. Characterization of polyethylene degradation within the mealworm gut. (a) Changes in molecular weight ($M_n$ and $M_w$) within residual polymer from the frass versus the control PE as measured by HT-GPC. Significance (Student’s t-tests, Tukey’s multiple test correction) $p < 0.05$ indicated by *, $p < 0.0005$ indicated by ***, no statistical significance indicated by ns. (b) Changes in the extractable fraction of the frass (% recovered by extraction), a measure of residual non-degraded and partially degraded polymer in the frass, over the 32-day experiment. (c) $^{1}$H-NMR spectra of residual polymer from the frass of PE and PE + bran fed mealworms versus residuals extracted from bran-fed mealworms and the control PE foam. The appearance of alkene derivatives is highlighted in grey. Detailed peak information in Table S2. (d) FTIR spectra of residual polymer from the frass of PE and PE + bran fed mealworms versus the control PE, annotations show functional groups associated with key peaks based on wavenumber. Detailed peak information in Table S3.
Figure 3. Microbial community analysis of gut microbiome in different diets. (a) Changes in community composition by family by diet (average from all replicates, Table S1), legend shows ten most abundant OTUs across all diets, un. indicates unclassified. (b) Inverse Simpson Index of the gut microbiome by diet. No statistical significance (ANOVA, Tukey’s multiple test correction) indicated by ns. (c) Principal Coordinate Analysis (PCoA) of microbial communities by diet based on Bray-Curtis distance, colored by diet and labeled with the sample ID (two outliers removed: PS1, PS_Bran2; PCoA with outliers Fig. S5).
**Figure 4.** Differential abundance analysis of gut microorganisms between experimental diets. OTUs shown significantly (BH adjusted $p < 0.05$) differed between diets. Direction of fold change ($\log_2$) indicates which diet each OTU is more strongly associated (labeled below x-axis).

(a) PE-fed microbiome versus bran-fed microbiome. (b) PE-fed microbiome versus PE + bran-fed microbiome. (c) PE-fed microbiome versus PS-fed microbiome. (d) PS-fed microbiome versus bran-fed microbiome.
Table 1. Characterization of PE and PS foams tested before and after degradation by mealworms (mean ± standard deviation, n = 4 for control samples, n = 3 for frass samples).

<table>
<thead>
<tr>
<th>Plastic</th>
<th>Color</th>
<th>Density (g/cm³)</th>
<th>Molecular Weight (Mₘ)</th>
<th>Molecular Weight (Mₐ)</th>
<th>Mₘ Reduction (%)</th>
<th>Mₐ Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyethylene (PE)</td>
<td>Clear</td>
<td>0.023 ± .0009</td>
<td>184590 ± 11343</td>
<td>27480 ± 7216</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PE Frass</td>
<td>--</td>
<td>--</td>
<td>71483 ± 9164</td>
<td>16462 ± 2330</td>
<td>61.27% ± 4.96%</td>
<td>40.10% ± 8.48%</td>
</tr>
<tr>
<td>PE + Bran Frass</td>
<td>--</td>
<td>--</td>
<td>88898 ± 17102</td>
<td>14397 ± 2334</td>
<td>51.84% ± 9.26%</td>
<td>47.61% ± 8.49%</td>
</tr>
<tr>
<td>Polystyrene (PS)</td>
<td>White</td>
<td>0.016 ± .0004</td>
<td>170741 ± 9797</td>
<td>88523 ± 5946</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PS Frass</td>
<td>--</td>
<td>--</td>
<td>137523 ± 13086</td>
<td>77229 ± 2790</td>
<td>19.46% ± 7.66%</td>
<td>12.76% ± 3.15%</td>
</tr>
<tr>
<td>PS + Bran Frass</td>
<td>--</td>
<td>--</td>
<td>136856 ± 12733</td>
<td>74724 ± 3820</td>
<td>19.85% ± 7.46%</td>
<td>15.59% ± 4.33%</td>
</tr>
</tbody>
</table>