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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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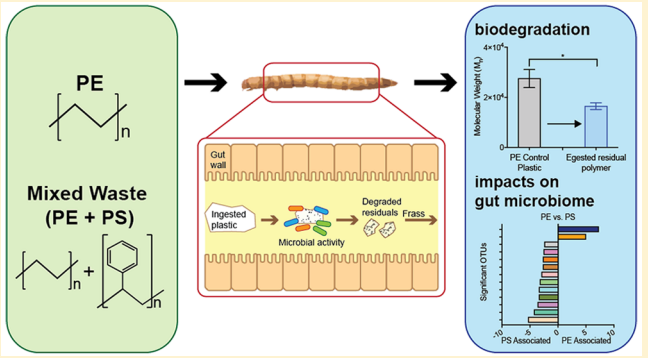
Supporting Information

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 nonspecific 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.[1−3] Plastic is of especially great concern in marine environments where its recalcitrance has led to accumulation and harmful effects on wildlife and potentially humans.[4,5] To combat this growing problem, there has been a steady increase in research on plastic biodegradation by bacteria and fungi.[6−8] 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.[6−8]

Recent work has demonstrated that mealworms (larvae of *Tenebrio molitor*), obtained from various sources across the globe, readily ingest and biodegrade polystyrene (PS) to CO_2 and lower molecular weight compounds within their gut.[9−11] Antibiotic studies implicated gut bacteria as agents of PS degradation, and *Exugiobacterium* sp. YT2, a bacterium capable of PS degradation was isolated from the gut.[10] Mealworms were shown to degrade nearly half of the ingested PS within the 12−15 h retention time in the gut, which is higher than the mass loss reported by the isolated *Exugiobacterium* sp. YT2 and other plastic-degrading bacterial isolates.[9,10,12] A recent report found that cofeeding PS with bran almost doubled the rate of PS degradation.[11]

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

To determine whether plastics may be broadly susceptible to biodegradation within mealworms, we evaluated the fate of

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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)

plastic	color	density (g/cm^3)	molecular weight (M_w)	molecular weight (M_n)	M_w reduction (%)	M_n reduction (%)
Polyethylene (PE)	clear	0.023 ± 0.009	184 590 ± 11343	27 480 ± 7216		
PE Frass			71 483 ± 9164	16 462 ± 2330	61.27% ± 4.96%	40.10% ± 8.48%
PE + Bran Frass			88 898 ± 17102	14 397 ± 2334	51.84% ± 9.26%	47.61% ± 8.49%
Polystyrene (PS)	white	0.016 ± 0.004	170 741 ± 9797	88 523 ± 5946		
PS Frass			137 523 ± 13086	77 229 ± 2790	19.46% ± 7.66%	12.76% ± 3.15%
PS + Bran Frass			136 856 ± 12733	74 724 ± 3820	19.85% ± 7.46%	15.59% ± 4.33%

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 homogeneous carbon-to-carbon backbone.[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 nonspecific. 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-h 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 h, 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.[11] Frass samples (50 mg) were gently crushed in a mortar and pestle prior to a 2-h 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 (≥99.9%, Thermo Fisher Scientific Inc., Pittsburgh, PA); for PS samples, the solvent was tetrahydrofuran (≥99.9%, Thermo Fisher Scientific Inc., Pittsburgh, PA) (Supporting Information, SI, M1, Figure S6). After 2 h, the solution was filtered using a 0.22 μm PVDF filter (Thermo Fisher Scientific Inc., Pittsburgh, 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

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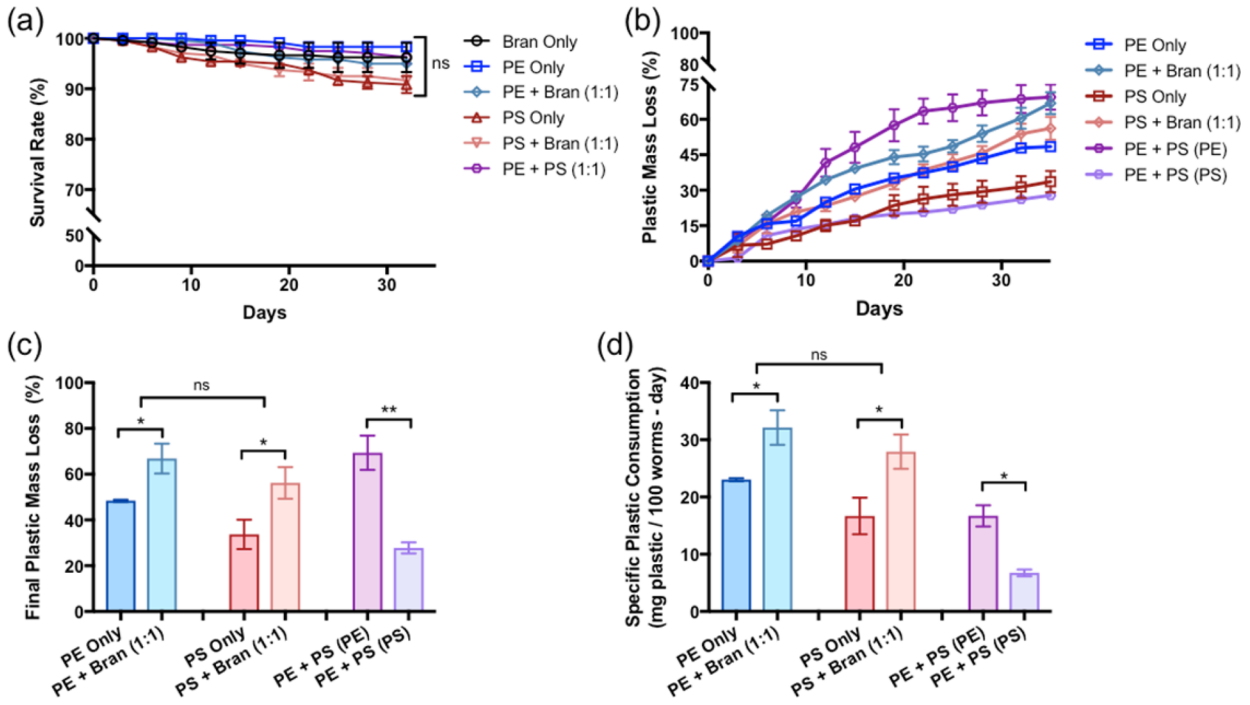

Figure 1. Survival rate and plastic consumption by *T. molitor* by diet. (a) Survival rate of mealworms over 32-day experiment. (b) Mass loss in plastic (PE or PS) in the plastic-fed diets over 32-days. (c) The percent mass loss in the plastic by diet at the end of the 32-day experiment. (d) Average 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.

recovered). Residual polymer was dissolved in 1,2,4-trichlorobenzene (≥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 uL 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 resuspended in chloroform-D (≥99.8%, Cambridge Isotope Laboratories, Inc., Tewksbury, MA). Proton-NMR spectra were obtained at 55 °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., Pittsburgh, PA). Spectra were recorded from the residual polymers extracted from the frass ground to a homogeneous 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., Pittsburgh, PA). Proton-NMR and FTIR analyses 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 μL of DNA extraction buffer (0.1 M NaH_2PO4, 0.1 M Na_2HPO4, 0.1 M EDTA, 0.1 M Tris-HCl, 1.5 M NaCl, and 1% CTAB).[18] 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.[19] Phasing amplicon sequencing was used to sequence the V4 region of 16S rRNA gene (SI M2).[20] 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 × 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.[21] 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).[22] 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

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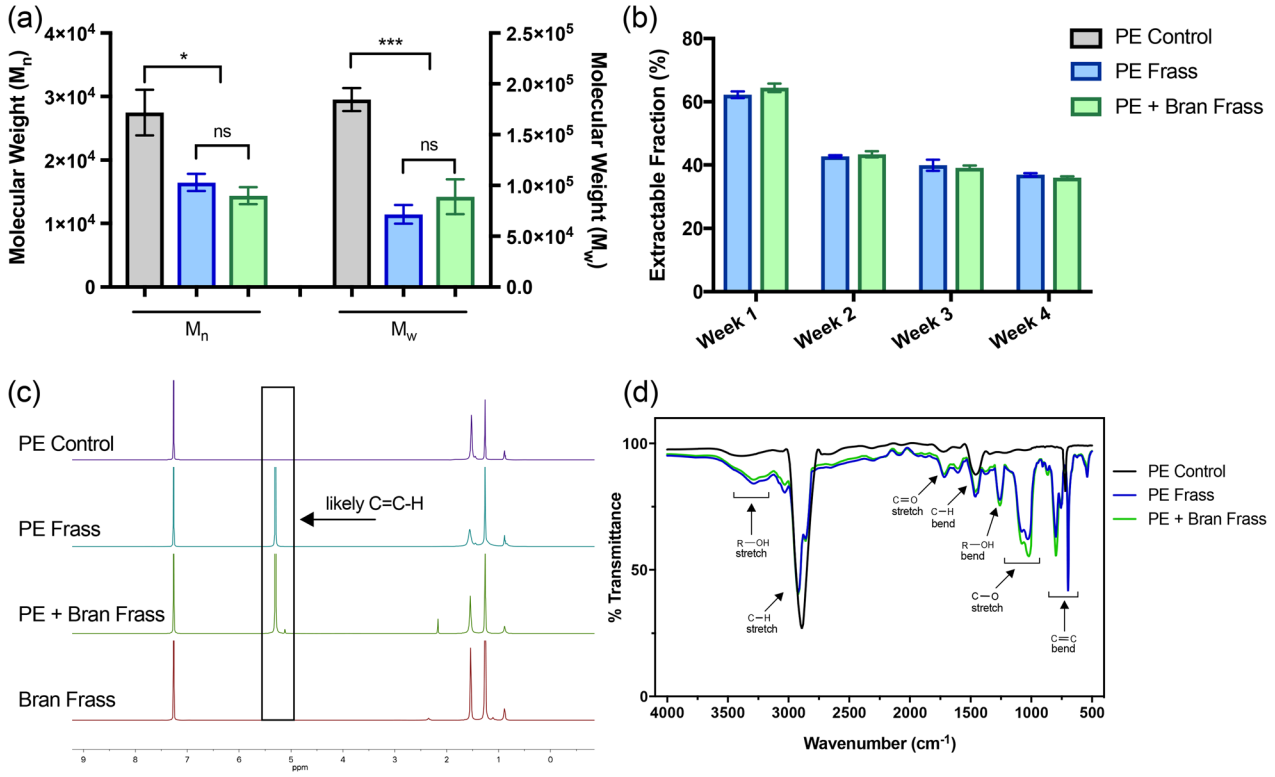

Figure 2. Characterization of polyethylene degradation within the mealworm gut. (a) Changes in molecular weight (M_n and M_w) within residual polymer from the frass versus the control PE as measured by HT-GPC. Significance (Student's t tests, Tukey's multiple test correction) $p < 0.05$ indicated by *, $p < 0.0005$ indicated by ***, no statistical significance indicated by ns. (b) Changes in the extractable fraction of the frass (% recovered by extraction), a measure of residual nondegraded 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 gray. 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.

threshold of 97% and singletons and chimeric sequences were removed.[23] Representative sequences of the OTUs were classified using RDP Classifier (training set 16, June 2016) with confidence cutoff of 0.8.[24] Diversity was evaluated using the R package "vegan."[25] Differential abundance analysis was conducted using the Bioconductor package DESeq2 in QIIME with the Benjamini-Hochberg (BH) correction for multiple testing.[26,27] 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%) (Figure 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%) (Figure 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: $p = 0.27$) (Figure 1a).[9,11]

Consumption of PE and PS increased throughout the experiment (Figure 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 (Figure 1c, d). For mealworms fed PS, the total PS mass loss was 0.57 ± 0.12 g (Figure 1c, d). For both PE- and PS-fed mealworms, the mass loss was significantly greater when the mealworms received bran as a cofeed. 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 (Figure 1c, d). Specific rates of plastic consumption (mg plastic consumed per 100 worms per day) followed the same pattern (Figure 1d). The increase in specific consumption when cofed with bran supports previous findings.[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 nondegraded and partially degraded polymer, and a nonextractable fraction (other biological waste not recovered in the extraction process).[11] A decrease in the extractable fraction ("residual polymer")

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suggests that more of the ingested plastic is being completely degraded (mineralized) and/or is being incorporated into mealworm biomass.[11] The extractable portion of the frass from mealworms fed PE or PS decreased over the course of the 32-day experiment (Figure 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 (Figure 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% (Figure 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% (Figure 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 (Figure S1a, Table 1). The observed depolymerization in mealworms fed PS and PS + bran is consistent with previous observations (Table 1).[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) (Figure 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 (Figure S1c, Table S2).[9,11] 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 h then subject to the same extraction methods (SI M3).[28] 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 (Figure S2a, b).[28]

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}) (Figure 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 (Figure 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 (Figure S2c, d, Table S3).[28]

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).[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 toward the end of the 32-day experiment (Figure 2b, Figure S3a). Mealworms fed plastic alone (PE or PS) gained less biomass weight over the course of the experiment than mealworms cofed with bran, an observation consistent with previous studies of PS-fed mealworms (Figure S3).[11] By mass balance, the PGF was 49.0 ± 1.4% at the end of the experiment for PE-fed mealworms (Figure 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 (Figure 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 ^{13}C-carbon tracing) (Figure S3c).[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 (Figure 1b, c, d). However, when mealworms were cofed PE and PS (1:1 [w/w]), there was a significantly higher mass loss of PE than PS (Figure 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.[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 (Figure S4, Table S3). This finding offers further evidence that plastic degradation within the mealworm gut is nonspecific.

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: *Spiroplasma*

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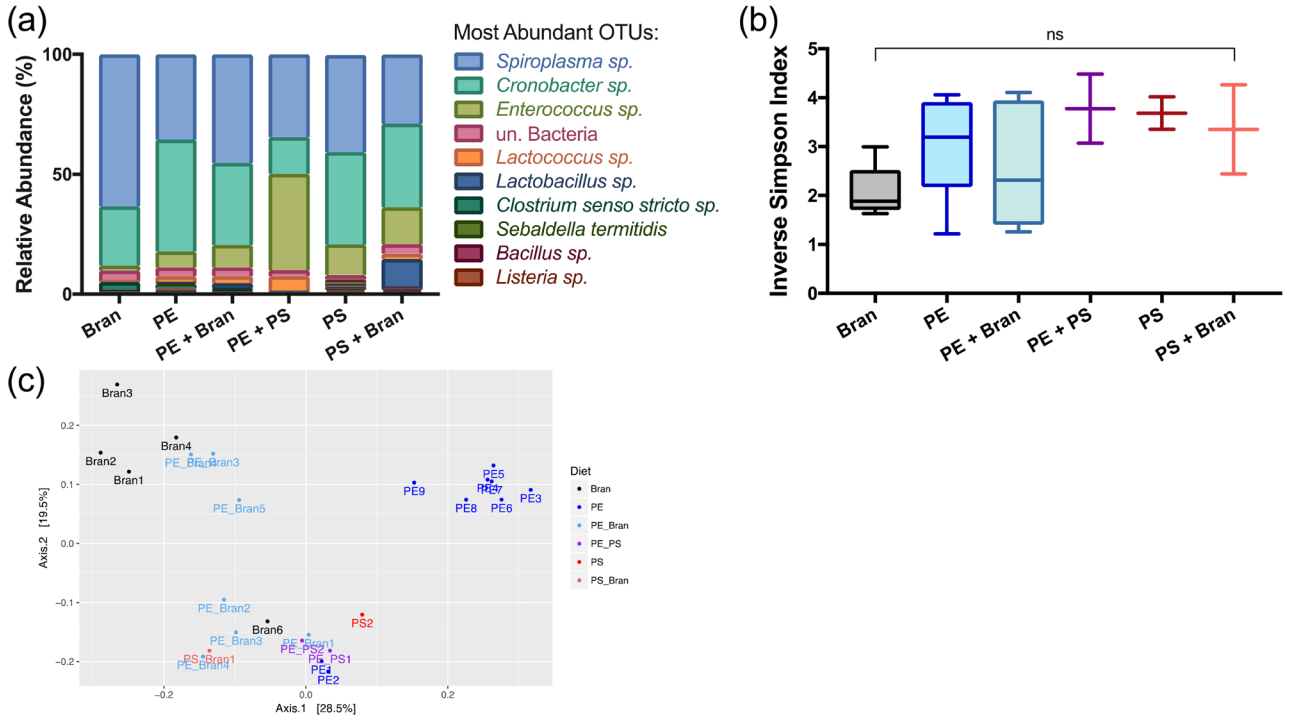

Figure 3. Microbial community analysis of gut microbiome in different diets. (a) Changes in community composition by family by diet (average from all replicates, Table S1), legend shows 10 most abundant OTUs across all diets, un. indicates unclassified. (b) Inverse Simpson Index of the gut microbiome 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 Figure S5).

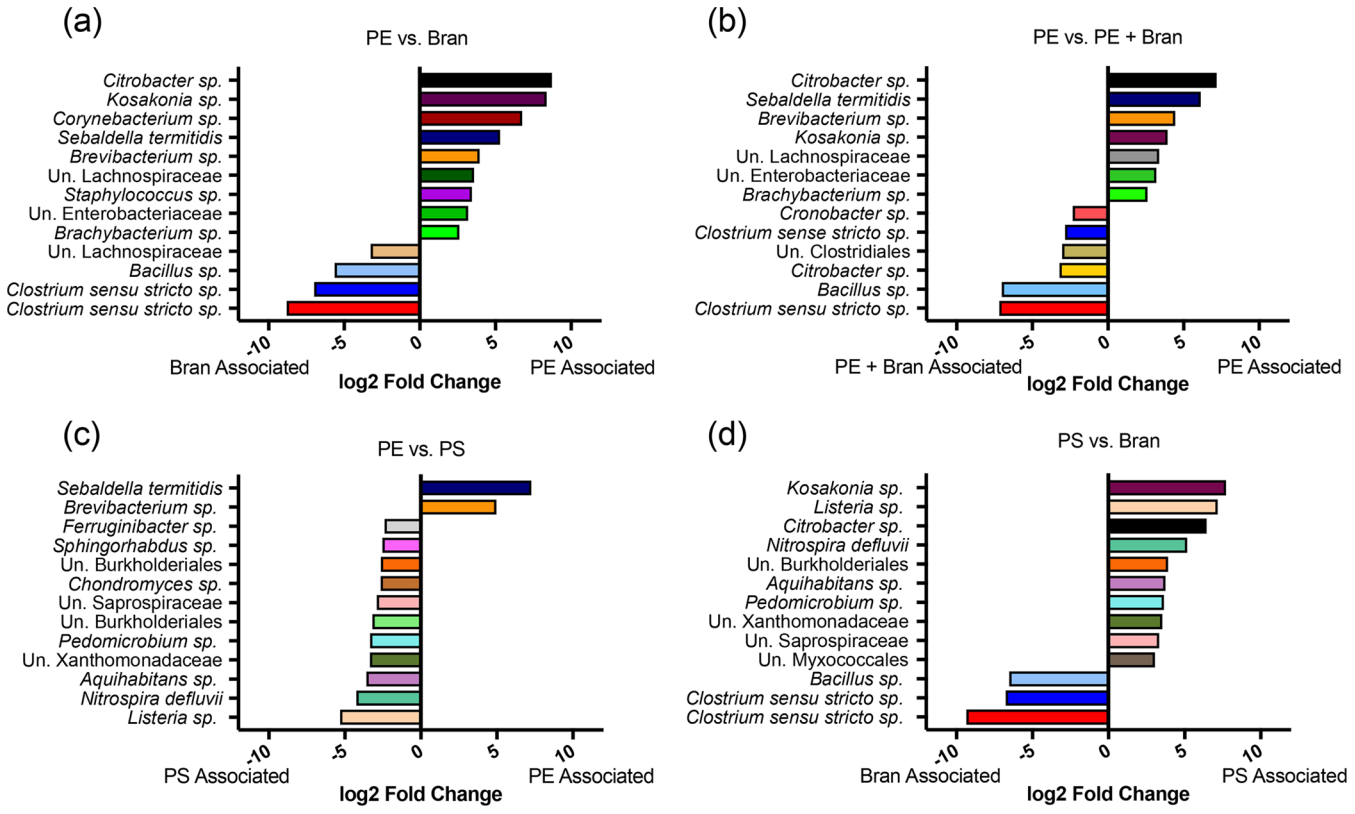

Figure 4. Differential abundance analysis of gut microorganisms between experimental diets. OTUs shown significantly (BH adjusted $p < 0.05$) differed between diets. Direction of fold change ($\log_2$) indicates which diet each OTU is more strongly associated (labeled below x-axis). (a) PE-fed microbiome 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.

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sp., *Cronobacter* sp., and *Enterococcus* sp. (Figure 3a). These three OTUs are common insect gut-associated bacteria and are known members of the *T. molitor* gut microbiome.[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 (Figure 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 (Figure 3c). A multiresponse 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 (Figure 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.[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 (Figure 2c, d; Figure S1c, d).[6−8,30] Both *Citrobacter* sp. and *Kosakonia* sp. were more abundant (based on relative abundance) in both of the plastic-only diets than those in 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. (Figure 4b−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 track.[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 (Figure 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

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b02301.

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 (PDF)

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The authors declare no competing financial interest.

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