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Confirmation of biodegradation of low-density polyethylene in dark- versus yellow- mealworms (larvae of *Tenebrio obscurus* versus *Tenebrio molitor*) via. gut microbe-independent depolymerization

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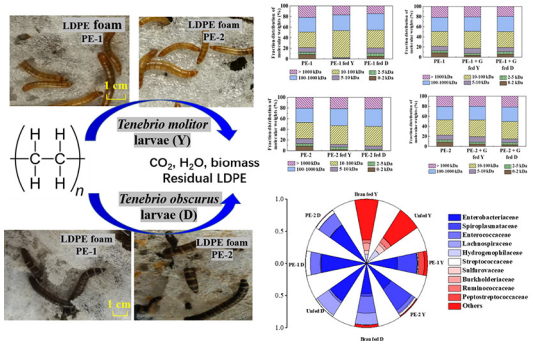
HIGHLIGHTS

- *Tenebrio obscurus* larvae biodegrading LDPE similar to *T. molitor*
- Biodegradation via broad depolymerization pattern with oxidized intermediates
- LDPE biodegradation is gut microbe independent but enhanced by microbes.
- Distinct gut communities developed and shaped with LDPE diets vs. origins.

GRAPHICAL ABSTRACT



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ABSTRACT

Tenebrio obscurus (Coleoptera: Tenebrionidae) larvae are capable of biodegrading polystyrene (PS) but their capacity for polyethylene (PE) degradation and pattern of depolymerization remains unknown. This study fed the larvae of *T. obscurus* and *Tenebrio molitor*, which have PE degrading capacity, two commercial low-density PE (LDPE) foams i.e., PE-1 and PE-2, with respective number-average molecular weights (M_n) of 28.9 and 27.3 kDa and weight-average molecular weights (M_w) of 342.0 and 264.1 kDa, over a 36-day period at ambient temperature. The M_w of residual PE in frass (excrement) of *T. obscurus*, fed with PE-1 and PE-2, decreased by 45.4 ± 0.4% and 34.8 ± 0.3%, respectively, while the respective decrease in frass of *T. molitor* was 43.3 ± 0.5% and 31.7 ± 0.5%. Data analysis showed that low molecular weight PE (<5.0 kDa) was rapidly digested while longer chain portions (>10.0 kDa) were broken down or cleaved, indicating a broad depolymerization pattern. Mass balance analysis indicated nearly 40% of ingested LDPE was digested to CO_2. Antibiotic suppression of gut microbes in *T. molitor* and *T. obscurus* larvae with gentamicin obviously reduced their gut microbes on day 15 but did not stop depolymerization because the M_n, Mw and size- average molecular weight (M_z) decreased. This confirmed that LDPE biodegradation in *T. obscurus* was independent of gut microbes as observed during previous PS degradation in *T. molitor*, suggesting that the intestinal digestive system could perform LDPE depolymerization.

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High-throughput sequencing revealed significant shifts in the gut microbial community during bran-fed and unfed conditions in response to LDPE feeding in both *Tenebrio* species. The respective predominant gut genera of *Spiroplasma* sp. and *Enterococcus* sp. were observed in LDPE-fed *T. molitor* and *T. obscurus* larvae.

1. Introduction

Petroleum based plastics have been widely used since the 1950s, with a global production of 359 million tons in 2018 (PlasticEurope, 2019). Major plastic polymers produced include polyethylene (PE), 29.7%; polypropylene (PP), 19.3%; polyvinylchloride (PVC), 10.3%; polyurethane (PUR), 7.9%; polyethylene terephthalate (PET), 7.7%; and polystyrene (PS), 6.4% (PlasticEurope, 2019). The most widely used polymer, PE, which is expressed as "$[CH_2-CH_2]_n$", is comprised of a linear carbon backbone. Commercial products made from this polymer include low-density PE (LDPE), linear low-density PE (LLDPE), medium density PE (MDPE), and high-density PE (HDPE). LDPE and LLDPE constitute about 17.5% of global plastic production, while MDPE and HDPE account for 12.2% (PlasticsEurope, 2019). Due to their highly stable C—C and C—H covalent bonds, high molecular weights, hydrophobic properties, and the absence of readily oxidizable and/or hydrolysable groups, PE is extremely resistant to biodegradation (Gautam et al., 2007). As a result, microplastics and nanoplastics which are largely comprised of PE are considered emerging environmental contaminants (Wu et al., 2017; Wang et al., 2021).

Research on PE biodegradation with microbial cultures has been reported since the early 1970s (Sen and Raut, 2015). Research results showed that PE-biodegradability by microorganisms or enzymes is limited even after pretreatment processes such as photo- or thermo-oxidation of polymers were applied (Hadad et al., 2005; Sudhakar et al., 2008; Watanabe et al., 2009; Nowak et al., 2011; Wilkes and Aristilde, 2017). However, recent studies revealed that PE can be rapidly biodegraded or digested in the larvae guts of members of the darkling beetle family (Coleoptera: Tenebrionidae) i.e., *Tenebrio molitor* (yellow mealworms) (Brandon et al., 2018; Yang and Wu, 2020; Yang et al., 2021a) and *Zophobas atratus* (superworms) (Peng et al., 2020a) in a matter of hours; and can be ingested and biodegraded by the larvae of some pest moths (Lepidoptera: Pyralidae) i.e., Indian mealmoths (*Plodia interpunctella*) (Yang et al., 2014), greater waxworms (*Galleria mellonella*) (Bombelli et al., 2017; Kong et al., 2019; Lou et al., 2020), and lesser waxworms (*Achroia grisella*) (Kundungal et al., 2019).

Darkling beetle is the common name of the family Tenebrionidae, the seventh-most speciose taxon in Order Coleoptera which comprises of approximately 20,000 species across 2300 genera worldwide (Slipinski et al., 2011). To date, six species (*T. molitor*, *T. obscurus, Z. atratus*, *Tribolium castaneum*, *Plesiophthalmus davidis,* and *Uloma sp.*) of darkling beetle have been confirmed to biodegrade PS plastics (Yang et al., 2015a; Peng et al., 2019; Peng et al., 2020a; Wang et al., 2021; Woo et al., 2020; Wu and Criddle, 2021; Kundungal et al., 2021), with *T.molitor* and *Z.atratus* additionally capable of degrading LDPE (Brandon et al., 2018; Yang et al., 2021b; Peng et al., 2020a). The larvae of commercial species *T. molitor*, *T. obscurus* and *Z. atratus* are attractive for their natural presence in forests with rotting wood (Calmont and Soldati, 2008) and known to consume dried leaves rich in lignocellulosic materials (Peng et al., 2019, 2020a). *T. molitor* and *Z. atratus* larvae sourced from China and the USA showed ubiquity in biodegradation of PS and LDPE (Yang et al., 2015a, b; Yang et al., 2018a, b; Yang et al., 2021a; Peng et al., 2020a; Yang and Wu, 2020). *T. molitor* larvae can also biodegrade polyvinyl chloride (PVC) (Peng et al., 2020b), polypropylene (Yang et al., 2021b), and hydrolyzable bioplastic polylactic acid (PLA) (Peng et al., 2021). In addition, *T. molitor* larvae were not negatively impacted by the additive hexabromocyclododecane (HBCD), a flame retardant in PS foam and other plastics, and did not accumulate HBCD in the biomass (Brandon et al., 2020).

Based on updated evidence, we hypothesized that members of darkling beetle which chew and ingest lignocellulosic materials, especially the *Tenebrio* genus, are capable of degrading major no-hydrolyzable and hydrolyzable plastics such as PS, LDPE, PP, PVC and PET. *Tenebrio* genus includes at least three morphologically different species, distinguished primarily by the color of their larvae i.e., yellow- (*T. molitor* Linnaeus 1758), dark- (*T. obscurus* Fabricius 1792), and brown- (*T. opacus* Duftschmid 1812) mealworms (Calmont and Soldati, 2008). *T. molitor* and *T. obscurus* have been reported worldwide (Peng et al., 2019) and commercially sold as a food source for pets, chicken and small animals in China, the USA, Europe and many other countries. In Europe, *T. molitor* is also authorized to be used for cattle, farmed fish and human consumption (EU Regulation 2017/89). Although the diet and life behavior of the yellow-colored *T. molitor* and larvae of *T. obscurus* differ (Peng et al., 2019), both have been observed to eat dried tree leaves. Previous research demonstrated that the larvae of *T. obscurus* are capable of biodegrading PS foam via gut microbe dependent depolymerization based on antibiotic suppression test, i.e. PS depolymerization stopped as the level of gut microbes dropped to nearly zero in the presence of the antibiotic gentamicin (Peng et al., 2019). The PS-degrading capacity of *T. obscurus* is similar to that of *T. molitor* larvae with gut-microbe dependence (Yang et al., 2015b; Yang et al., 2018b). However, it remains unknown whether *T. obscurus* has a similar LDPE-degrading capacity to *T. molitor*, and if this ability is ubiquitous in the *Tenebrio* genus.

Biodegradation of non-hydrolysable plastics such as PE involves breaking down polymer chains (depolymerization) followed by the degradation and mineralization of intermediates to CO_2 and H_2O (Yang et al., 2014). This first and essential reaction of depolymerization is commonly characterized using gel permeation chromatography (GPC) based on changes in the number-average molecular weight (M_n), weight-average molecular weight (M_w), size-average weight (M_z) and molecular weight distribution (MWD) of polymers. The formation of oxidized functional groups is usually verified using analysis by Fourier-transform infrared spectroscopy (FTIR), ^{1}H nuclear magnetic resonance (^{1}H NMR) and other analytical methods (Yang et al., 2018b; Peng et al., 2020a, b; Wu and Criddle, 2021). The role of gut microbes in plastic degradation is commonly characterized by monitoring depolymerization while inhibiting their function using antibiotics. If depolymerization is inhibited with the suppression of gut microbes, the biodegradation is regarded as gut-microbe dependent, otherwise, it is considered to be independent of gut microbes (Yang et al., 2015b; Yang et al., 2018a; Peng et al., 2019; Peng et al., 2020a; Yang et al., 2021a, b).

The objectives of this study were to verify the capacity of LDPE biodegradation in *T. obscurus* larvae in comparison to *T. molitor* larvae, which are known to be capable of degrading PE, and to characterize the depolymerization/biodegradation in the larvae. The results indicated that *T. obscurus* larvae did consume and biodegrade two commercial LDPE foams, which supported our hypothesis of LDPE-degrading capability in the larval gut of the *Tenebrio* genus; and also indicated that both species perform gut-microbe independent LDPE depolymerization with significant shifts in gut microbiome as LDPE was degraded.

2. Materials and methods

2.1. Mealworm sources

Tenebrio molitor larvae with an average weight of 27 ± 2.5 mg/ larva were purchased from a local mealworm farm in Harbin, China (Graphic

abstract). *Tenebrio obscurus* larvae (approximately 130 ± 5.0 mg/larva in average weight) were purchased from Zaozhuang breeding farm in Shandong, China (Graphic abstract, Table 1). Prior to experiments, the larvae were fed natural wheat bran. The elementary ratio of the bran was: C:H:O:N:S (*w*/w) at 41.35:6.28:43.65:2.69:0.26. The larvae of both species were not previously fed with any antibiotics according to the suppliers. After stabilization, they were subject to a 48-h starvation period to empty their guts before initiation of the experimental diets.

2.2. PE foams

Two commercial LDPE foams, named as PE-1 (0.5 mm thickness, density mg/cm^3) and PE-2 (5.0 mm thickness, density mg/cm^3), were purchased from an online vender (Kexinda polymer materials, Shanghai, China) and used as feedstocks for the larvae (Graphic abstract). The M_n of PE-1 was 28,900 ± 1000 and M_w was 342,000 ± 13,000 with a density of 0.017 g/cm^3 and polymer diversity index (Polydispersity index (PDI) = M_w / M_n) of 11.8. PE-2 had M_n and M_w of 27,300 ± 1100 and 264,000 ± 11,900, respectively with a density of 0.024 g/cm^3 and PDI of 9.7. According to the manufacturer, no extra plasticizer additives were present. Both are commonly used LDPE foams and PE-1 contained more long chain polymers than PE-2.

2.3. Biodegradation capability of commercial PE by both mealworms sources

The biodegradability of the two commercial LDPE foams was tested using the larvae of both *Tenebrio* species. The larvae of *T. molitor* (n = 500, average weight 26.8 ± 0.3 mg per larva) and *T. obscurus* (n = 500, average weight 131.2 ± 3.2 mg per larva) were incubated in respective food grade polyethylene storage containers, with the rearing density of ~2 larvae/cm^2.

To assess the LDPE consuming capacity and biodegradation of the larvae, a total of eight treatments were performed: a) unfed *T. molitor*, b) PE-1 fed *T. molitor*, c) PE-2 fed *T. molitor*, d) bran fed *T. molitor*, e) unfed *T. obscurus*, f) PE-1 fed *T. obscurus*, g) PE-2 fed *T. obscurus*, and h) bran fed *T. obscurus*. PE-only fed treatments (i.e., PE-1 fed *T. molitor*, PE-2 fed *T. molitor*, PE-1 fed *T. obscurus*, and PE-2 fed *T. obscurus*, respectively) were initiated with the addition of 1.95 ± 0.05 g PE foam. The residual PE foam was weighed every six days to calculate PE consumption. Each incubator with 500 larvae was supplied with 1.95 ± 0.05 g of PE-1 or PE-2 foam which was cut into 5–6 cm irregular sized pieces. The larvae fed bran alone and unfed (starvation) served as controls. Bran-fed treatments (i.e., bran fed *T. obscurus* and bran fed *T. molitor*) were initiated by the addition of 3.6 g wheat bran followed by an additional 3.6 g bran every six days. All treatments were performed in triplicates. During the incubation, the containers were maintained in a 250 L artificial climate chamber (Artificial climate incubator, Shanghai Shuli, Shanghai, China) at 25 ± 0.5 °C and 65 ± 5% humidity.

After start-up tests, the mass of residual LDPE foam was weighed to calculate PE consumption every 6 days. Survival rates (SRs) of the larvae were calculated based on the number of live larvae versus the number of initially inoculated larvae. The LDPE consumption and SRs were monitored until day 36. During this 36-day period, no pupae were observed, similar to observations with LDPE as the sole diet for *T. molitor* (Brandon et al., 2018). Dead larvae were counted and removed from the containers to prevent the larvae from eating dead bodies, although cannibalism still occurred during the incubation period as observed previously (Peng et al., 2019). At the end of the test, the larvae were cleansed with compressed air to remove residual LDPE debris, then transferred to a clean PP container for 12 h to collect frass. The frass samples were stored at −20 °C for further analysis. For DNA analysis of the gut microbes, larval samples (at least 20) were collected at the end of 36-day test, preserved in 100% ethanol (analytical grade) and stored at −80 °C.

2.4. Characterization of LDPE biodegradation with egested frass

Biodegradation of LDPE in the larvae was characterized by determining any physical and chemical modifications, changes in chemical structure, and biodegraded intermediates and products (Wu and Criddle, 2021). Depolymerization of LDPE was characterized by collecting frass samples and analyzing residual PE polymers to determine M_n, M_w and M_z as well as MWD with high-temperature gel permeation chromatography (HT-GPC, PL GPC 220, Agilent Technologies, Inc., USA). Frass samples (100 mg) for each treatment group were collected, dried and gently crushed in a mortar and pestle. Extraction of residual PE from the samples was conducted by dissolving PE in 4 mL of 1,2,4-trichlorobenzene (1,2,4 TCB) solvent (Honeywell International, Inc., USA) followed by a 4-h heating period to facilitate extraction. The extracted solvent was filtered through an original stainless-steel filter and transferred into a clean glass vial. The extracted polymer was dried, weighed and redissolved in 1,2,4-TCB solvent to obtain a final concentration of 1.2 mg/mL prior to injection into HT-GPC. The analysis of each sample was performed at 150 °C using a 200 μL injection volume with an eluent (1,2,4 TCB) flow rate of 1.0 mL/min. PDI was calculated as PDI = M_w / M_n.

Fourier Transform Infrared Spectroscopy (FTIR) analysis (Nicolet iS50 FTIR Spectrometer, Thermo Fisher Scientific, U.S.A.) was used to characterize the major functional groups in the frass of LDPE fed larvae versus respective LDPE feedstocks. Frass samples were ground with potassium bromide (KBr) into homogeneous powders for scanning and graphing with FTIR. The spectra of all samples were obtained in absorbance mode and in the spectral region of 400–4000 cm^{-1} using a resolution of 0.6329 cm^{-1} and a minimum of 16 scans.

^{1}H NMR analysis was performed to characterize the formation of oxidized functional groups in the residual polymers extracted from frass samples. The control LDPE samples were PE-1 and PE-2 foams. Frass samples (10 mg) of LDPE-fed larvae were gently crushed in a mortar

Table 1
Characteristics of two different commercial PE foams after consumption and biodegradation by *T. molitor* and *T. obscurus* larvae.

Mealworm source	Feedstocks	Initial weight, mg larva^{-1}	Cumulative PE consumption, mg	SR, %	Specific PE consumption[a]		M_n of frass (kDa)	M_n reduction (%)	M_w of frass (kDa)	M_w reduction (%)	PDI
					SPEC-N, mg PE·100 larvae^{-1} d^{-1}	SPEC-W, mg PE·g larvae^{-1} d^{-1}					
T. molitor	Unfed	25.9 ± 0.4	nd	71.7 ± 0.6	Nd	nd	nd	nd	nd	nd	nd
	Bran	27.3 ± 0.6	nd	96.1 ± 0.5	Nd	nd	nd	nd	nd	nd	nd
	PE-1	25.9 ± 0.2	882.0 ± 9.9	95.6 ± 0.4	5.0 ± 0.7	2.6 ± 0.4	26.7 ± 0.7	7.6 ± 0.9	193.8 ± 9.1	43.3 ± 0.5	7.3
	PE-2	28.1 ± 0.1	854.4 ± 7.7	95.5 ± 2.2	4.9 ± 0.3	2.4 ± 0.1	24.3 ± 1.1	11.0 ± 0.2	180.3 ± 9.4	31.7 ± 0.5	7.4
T. obscurus	Unfed	131.3 ± 3.1	nd	74.3 ± 1.9	Nd	nd	nd	nd	nd	Nd	nd
	Bran	131.6 ± 4.1	nd	90.4 ± 0.7	Nd	nd	nd	nd	nd	Nd	nd
	PE-1	132.7 ± 3.3	774.4 ± 17.3	88.6 ± 0.5	4.5 ± 0.4	0.4 ± 0.0	25.9 ± 1.0	10.4 ± 0.3	186.8 ± 8.5	45.4 ± 0.4	7.2
	PE-2	129.2 ± 2.3	585.2 ± 13.2	91.6 ± 0.7	3.4 ± 0.3	0.3 ± 0.0	25.5 ± 0.9	6.6 ± 0.7	172.2 ± 8.5	34.8 ± 0.3	6.8

Note: The initial number: Y = *T. molitor* larvae, 500; D = *T. obscurus* larvae, 500. nd = not determined.
[a] Specific PE consumption was calculated on the basis of the mass of PE consumed over the test period (36 days) and the initial number of larvae.

and dissolved in 1 mL chloroform-D solvent (D, 99.8%, Cambridge Isotope Laboratories, Inc., Andover, U.S.A.) in a 10 mL glass bottle. The glass bottle was heated for 4-h at 55 °C in a thermostat water bath. After heating extraction, the extracted residual polymer was resuspended in chloroform-D and analyzed by using a Bruker Avance NEO 600 spectrometer (Bruker Corporation, Germany). The ^{1}H-spectra [16 scans, delay time (d1) = 1.0 s] were referenced to the residual deuterated-chloroform peak [7.26 ppm]. Spectra were analyzed using MestReNova software (v12.0.1). ^{1}H NMR analyses were run in triplicates for samples of both LDPE foams and residual polymer samples.

Biodegradation of LDPE is indicated by a decrease in stability. LDPE foams (PE-1 and PE-2) and frass samples containing residual LDPE were analyzed by TGA (TGA/SDTA851e, Mettler Toledo, U.S.A.). Samples were heated from 30 to 900 °C at a rate of 10 K/min under a high-purity nitrogen ambience (99.999%).

2.5. Carbon mass balance analysis

Test of the carbon mass balance for the ingested LDPE by *T. molitor* and *T. obscurus* larvae was conducted using batch trails with incubators equipped with a four- stage CO_2 trapping system modified from that described by Yang et al. (2015a). The larvae (200 per incubator) were incubated in glass jars (1000 mL in volume) and fed a sole diet of their respective PE-1 and PE-2 foams (0.5 g) with triplicates (Fig. S3). An additional two jars without the larvae served as the control. Released CO_2 was trapped in a 2 M NaOH solution and carbon in CO_2 was recovered as $CaCO_3$ precipitate by adding $CaCl_2$. LDPE in frass was extracted using an olive oil-based method developed by Scopetani et al. (2020). The carbon balance analysis was conducted to measure the carbon mass of ingested LDPE, PE residue in frass, CO_2 released from biodegradation, and increment of carbon in biomass in addition to estimating the digestibility of the ingested LDPE, carbon conversion rate of digested LDPE to CO_2, and the ratio of the carbon assimilated to biomass after biodegradation. The measurement was conducted after 18 days incubation. The detailed procedures and calculation are described in Supporting information.

2.6. Antibiotic suppression test

The effect of antibiotic suppression on PE depolymerization and biodegradation was tested using the larvae of *T. molitor* and *T. obscurus* larvae with gentamicin sulfate (Yang et al., 2015b; Yang et al., 2018a; Peng et al., 2019; Peng et al., 2020b). The selection of antibiotics was re-examined prior to antibiotic suppression tests in this study. Six different antibiotics (gentamicin, chloramphenicol, erythromycin, kanamycin sulfate, penicillin, and tetracycline) were screened for their ability to inhibit gut bacteria from *T. molitor* larvae based on inhibition halo tests (Figs. S2, SI Section S2 in Supporting information) described by Visôtto et al. (2009) and Yang et al. (2015b). The gut suspension (100 μL) was inoculated and spread across an Luria-Bertaini (LB) medium plate (Wu and Criddle, 2021). Subsequently, the antibiotic discs of the six aforementioned antibiotics (30 μg antibiotics per disc) were placed on the surface of the inoculated plates. The discs containing no antibiotic served as the control. The plates were incubated in triplicates for 24 h at 37 °C, and then the inhibition halos were measured. The antibiotic resulting in the biggest inhibition halo was selected for suppressing the gut bacteria.

LB medium was prepared with DI water containing (per 1000 mL) 10 g bacteriological tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar. All media were sterilized by autoclaving at 121 °C for 30 min.

Two gentamicin suppressive groups (500 larvae) were fed antibiotic feedstocks containing bran plus gentamicin sulfate 100:3 (w/w) for 15 days and then fed with PE-1 and PE-2 feedstocks, respectively. On day 0, 7, and 15, larvae gut samples (20 each) were randomly selected from each diet group to prepare a gut suspension for counting of the gut microbes. The larval specimen was decontaminated with 75% ethanol, rinsed by Milli-Q water, and anatomized to obtain guts for further microbial community analyses.

Gut contents were extracted and suspended in 5 mL sterile saline water, serial diluted (10^{-1} to 10^{-7}) and inoculated on nonselective TSA plates at 37 °C for 24 h. The procedure was the same as described previously (Yang et al., 2018a). After day 15, antibiotic feedstock was continuously fed together with LDPE diet and resupplied every 2 days during LDPE feeding period. Frass samples from the antibiotic treatment were collected to analyze the molecular weights using HT-GPC analysis.

2.7. Microbial community analysis

To compare the changes in gut communities of *T. molitor* and *T. obscurus* larvae before and after feeding LDPE, 20 larvae from each *T. molitor* and *T. obscurus* group fed with the two LDPE foams (PE-1and PE-2) were randomly selected for microbial community analyses at the beginning and the end of the 36-day experiment. All treatments in the tests were performed in triplicates. Samples used for DNA extraction included the larvae fed each LDPE, bran, and the unfed larvae which were euthanized and preserved in 80% alcohol. Subsequently, the larval body was washed with sterile deionized water three times. After dissection, the intestinal tissues with ingested/digested contents were collected and stored in 100% ethanol (analytical grade) at −80 °C prior to use. All DNA from the larval gut plus a small amount of frass that remained in the gut was extracted for microbial community analysis. Total bacterial genomic DNA samples were extracted using Fast DNA SPIN extraction kits (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The DNA extraction and PCR amplification methods were similar to that reported previously (Lou et al., 2020). Phasing amplicon sequencing was applied to sequence the V3 − V4 region of the 16S rRNA gene. Purified amplicons were paired-end 2 × 300 bp sequenced on an Illumina MiSeq platform with MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The low-quality sequences were filtered through following criteria (Gill et al., 2006; Chen and Jiang, 2014) i.e., length < 150 bp, average Phred scores <20, containing ambiguous bases, and containing mononucleotide repeats >8 bp. Operational Taxonomic Units (OTUs) were clustered with 0.97 identity threshold using UPARSE (v9.2.64). Chimeric sequences were identified and removed using UCHIME (v4.2.40). Taxonomy of each 16S rRNA gene sequence was analyzed by Ribosomal Database Project (RDP) Classifier against the Silva 16S rRNA database with a confidence threshold of 70%. Sequence data analyses were mainly performed using QIIME (v1.9.1) and R packages (v3.4.3) including OTU-level alpha diversity analysis, microbial community composition analysis, principal component analysis (PCA) and hierarchical clustering analysis.

2.8. Statistical analysis

Statistical analyses were conducted to evaluate the differences in PE consumption, survival, changes in molecular weights, antibiotic suppression on PE depolymerization, and microbial diversity. ANOVAs were performed, followed by pairwise comparisons using Student's *t*-test with Tukey's correction to assess significance. Statistical analyses were performed in IBM SPSS Statistics (v25). All *p*-values are adjusted *p* values and all error values are average ± standard deviation.

3. Results and discussion

3.1. LDPE foam consumption by T. obscurus vs T. molitor larvae

The larvae of both *T. molitor* and *T. obscurus* were able to chew and eat LDPE foams (Graphic abstract). *T. obscurus* larvae behaved similarly to *T. molitor* larvae on ingestion of both the LDPE foams but were more sensitive to light than *T. molitor* larvae and mostly hid below the foams as also observed previously during PS biodegradation with *T. obscurus*

larvae from other sources in China and the USA (Peng et al., 2019). The mass of consumed LDPE foams increased progressively during the test period (Fig. 1c). By the end of test, of the initial amount of 1.95 ± 0.05 g LDPE foam, the total LDPE mass consumed by *T. molitor* larvae was 882.0 ± 9.9 mg and 854.4 ± 7.7 mg for PE-1 and PE-2, respectively; while *T. obscurus* larvae consumed 774.4 ± 17.3 mg and 585.2 ± 13.2 mg of PE-1 and PE-2, respectively (Fig. 1a, Table 1). The change in SRs of both *Tenebrio* species fed on PE foams over the 36 days rearing period is depicted in Fig. 1b. At the end of 36-day experiment, the SRs of *T. molitor* fed with PE-1 and PE-2 foams as the sole diet was 95.6 ± 0.4% and 95.5 ± 2.2% respectively; while SRs of *T. obscurus* larvae fed with PE-1 and PE-2 was 88.6 ± 0.5% and 91.6 ± 0.7%, respectively (Table 1). The SRs of the bran-fed *T. molitor* larvae was 96.1 ± 0.5% and that of the *T. obscurus* larvae was 90.4 ± 0.7%. The results indicated that the SR of the larvae fed with LDPE foams were statistically similar to those fed with bran during the 36 days. However, *T. molitor* larvae had slightly higher SR than *T. obscurus* larvae regardless of whether they were fed bran or LDPE foams (Fig. 1b and Table 1). For unfed groups, SR was only 71.7 ± 0.6% and 74.3 ± 1.9% for *T. molitor* and *T. obscurus* larvae, respectively, significantly ($p < 0.01$) lower than those of the two PE-fed *T. molitor* larvae (PE-1: 95.6 ± 0.4%; PE-2: 95.5 ± 2.2%) and *T. obscurus* larvae (PE-1: 88.6 ± 0.5%; PE-2: 91.6 ± 0.7%), indicating that the larvae ingested LDPE foams and maintained activities for their survival. This was also observed previously when the larvae of both

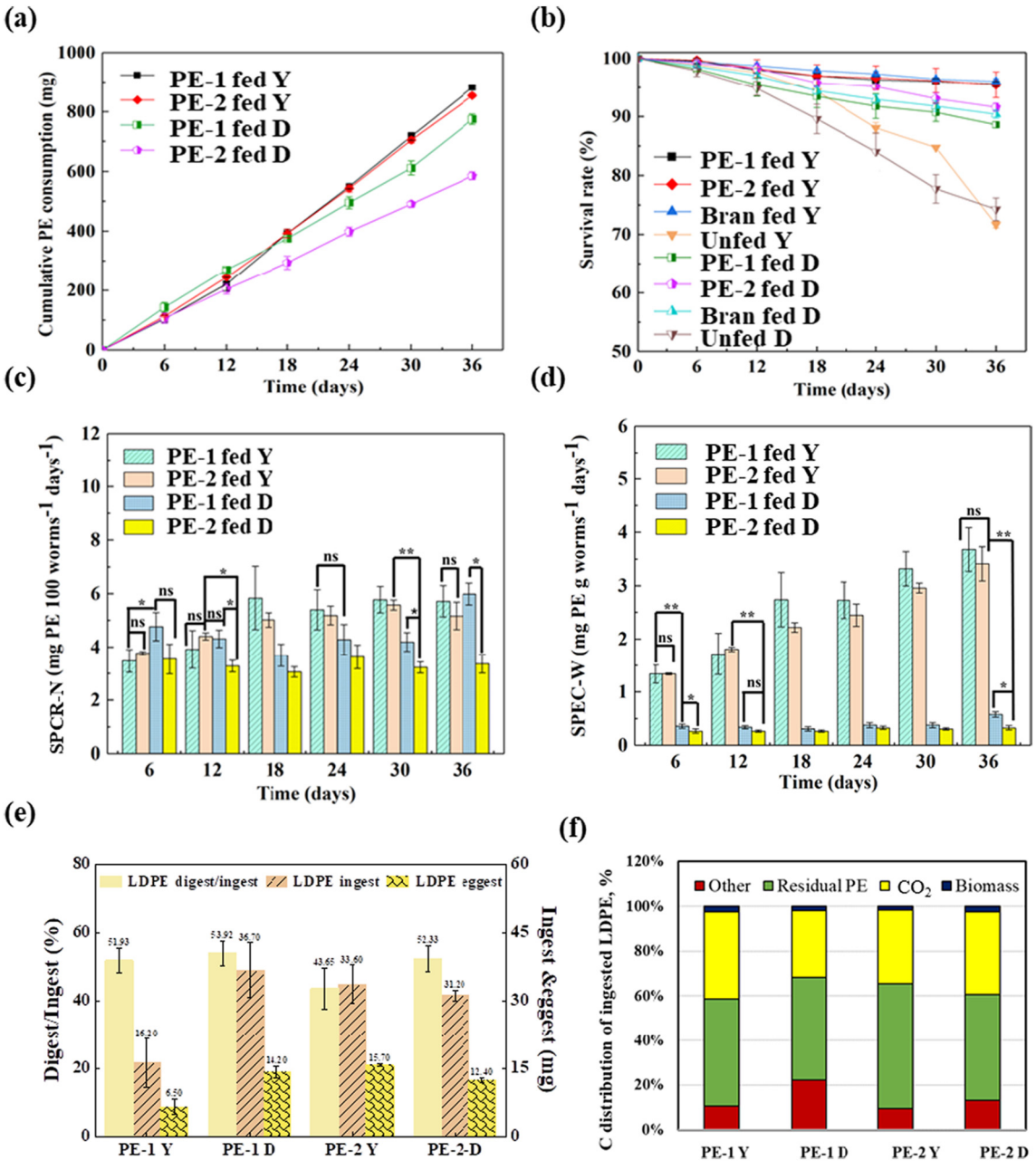

Fig. 1. Ingestion and consumption of LDPE by *T. molitor* and *T. obscurus* larvae. (a) Comparison of PE-1 and PE-2 consumption by *T. molitor* and *T. obscurus* larvae fed with LDPE only. (b) Survival rates of the larvae fed with PE-1 and PE-2 only, bran versus unfed. (c) Specific LDPE consumption rates based on 100 larvae (SPEC-N, mg PE·100 larvae^{-1} d^{-1}) of the larvae fed with respective PE-1 and PE-2 only over 36 days. (d) Specific PE consumption rates based on larval weights (SPEC-W, mg PE·g larvae^{-1} d^{-1}). (e) The digestibility or the ratio of digested LDPE versus ingested LDPE and the mass of ingested versus egested LDPE by 200 larvae over 18 days period. (f) Carbon distribution of ingested LDPE to biomass, CO_2, residual LDPE in frass and other intermediates. Y = *T. molitor* larvae; D = *T. obscurus* larvae. (All values represent mean ± SD, $n = 3$. Significance (Student's *t*-tests) $p < 0.05$ indicated by *, $p < 0.01$ indicated by **, and no statistical significance indicated by ns.)

species ingested PS as the sole diet during a short-term experiment (4–5 weeks) (Yang et al., 2015a; Yang et al., 2018a; Peng et al., 2019). According to literature and our observation, although *T. obscurus* and *T. molitor* belong to the same genus, they are different in their behaviors. *T. obscurus* larvae were more sensitive to light, difficult to breed and frequently die before pupation (Fraenkel, 1956), and have high cannibal characteristics (Peng et al., 2019). Thus, the relatively lower SR of *T. obscurus* larvae as compared to *T. molitor* larvae was observed regardless the diet.

Specific LDPE consumption rates (SPCR, mg PE 100 larvae^{-1} d^{-1}) were calculated based on the LDPE mass consumed by 100 larvae over the test period using the average number of live larvae (Wu and Criddle, 2021). Over 36-day, SPCRs of PE-1 and PE-2 for *T. molitor* larvae were 5.0 ± 0.7 and 4.9 ± 0.3 mg·100 worms^{-1} d^{-1}, respectively; and SPCRs of PE-1 and PE-2 for *T. obscurus* larvae were 4.5 ± 0.4 and 3.4 ± 0.3 mg·100 larvae^{-1} d^{-1} respectively (Fig. 1c and Table 1). Although both species showed similar LDPE consumption rates, the *T. molitor* larvae had slightly higher LDPE SPCRs than *T. obscurus* because *T. obscurus* larvae used in this study had larger average size and weight than that of the *T. molitor* larvae (Graphic abstract). Based on the average weight of the larvae, the specific LDPE consumption rate on larval weight basis (SPCR-W, mg PE g larvae^{-1} d^{-1}) of *T. obscurus* larvae was much lower than that of *T. molitor*. The SPCR-W of PE-1 was 2.6 ± 0.4 mg LDPE·g larvae^{-1} d^{-1} and PE-2 was 2.4 ± 0.1 mg LDPE·g larvae^{-1} d^{-1} for *T. molitor* larvae while the respective rates for *T. obscurus* larvae were 0.4 ± 0.0 mg LDPE·g larvae^{-1} d^{-1} and 0.3 ± 0.0 mg LDPE·g larvae^{-1} d^{-1} (Fig. 1d and Table 1). Chewing by the larvae enhanced fragmentation of LDPE film, resulting in an increased contact area for LDPE with the gut microbial consortia and enzymes. The insect's alimentary tract is split into three main parts: the foregut (stomodeum), midgut (mesenteron), and hindgut (proctodeum) (Chapman, 2013). The larval gut can be considered an efficient bioreactor. Gut microbial consortia and gut enzymes are likely critical for the rapid LDPE biodegradation in the bioreactor. Further study is needed to understand whether the difference in the consumption rates between the larvae of the two species tested in this study is generic or larval source specific (i.e., insect mouth feeders, stretch receptors, body wall, and alimentary canal), since the eating habits of the two species are different (Makkar et al., 2014).

3.2. LDPE depolymerization

LDPE depolymerization and biodegradation through the digestive intestine of *T. molitor* and *T. obscurus* larvae was characterized using HT-GPC analysis (Fig. 2). Both PE-1 and PE-2 were significantly depolymerized by the larvae of *T. obscurus* and *T. molitor* based on the molecular weight changes and MWD. It was observed that the MWD shifted towards lower molecular weights after the LDPE polymers were partially digested and egested in frass (Fig. 2a and b). For PE-1 fed larvae of *T. molitor* and *T. obscurus*, M_n was reduced by 7.6 ± 0.9% and 10.4 ± 0.3% respectively; M_w was reduced by 43.3 ± 0.5% and 45.4 ± 0.4%, respectively, and M_z was reduced by 45.0% and 50.1%, respectively. For PE-2 fed larvae of *T. molitor* and *T. obscurus*, M_n was reduced by 11.0 ± 0.2% and 6.6 ± 0.7%; M_w dropped by 31.7 ± 0.5% and 34.8 ± 0.3%, and M_z declined by 37.2 ± 0.5% and 46.0 ± 0.4%, respectively (Fig. 2c and d). Results showed significant reductions ($p < 0.05$) in M_n, M_w and M_z of PE-1 and PE-2 after passage through the digestive tract of the larvae of both species, confirming that both performed broad depolymerization (Table 2). From the results obtained in this study, the extent of the decrease in M_n, M_w, and M_z of the two LDPE foams by *T. obscurus* larvae were higher than that of *T. molitor* larvae, similar to observations of PS degradation by *T. obscurus* versus *T. molitor* (Peng et al., 2019). This suggested that the *T. obscurus* larvae tested might have higher plastic polymer digestibility than *T. molitor* larvae.

The fractional distribution of molecular weight of the two LDPE foams and the corresponding LDPE residues extracted from the frass of *T. molitor* and *T. obscurus* larvae were analyzed for comparison in Fig. 2e and f. Frass samples from the *T. molitor* and *T. obscurus* fed with PE-1 only contained <0.2% and 4.99% of small sized polymer <2.0 kDa while raw PE-1 foam contained 8.52% of the small sized polymer (Fig. 2e). Similarly, the residual polymers in the frass from *T. molitor* and *T. obscurus* fed with PE-2 contained 1.85% and 0.29% of the small polymer, respectively (Fig. 2f, Table S3), which was significantly lower than 8.50% of the small polymer in PE-2 foam. Higher molecular fractions >1000 kDa detected in the frass samples from *T. molitor* and *T. obscurus* larvae were also reduced (Fig. 2e and f). However, the fraction of polymer with molecular weight between 10.0 and 100 kDa were higher in the frass samples from both *T. molitor* and *T. obscurus* larvae, suggesting their accumulation during biodegradation. Previous research on pretreatments using thermo-oxidative PE degradation with pro-oxidant revealed that reduction of the average molecular weight to below 5.0 kDa was the most important step to achieve significant biodegradation (Jakubowicz, 2003; Koutny et al., 2006) because low molecular weight fractions (<5.0 kDa) can be rapidly decomposed (Chiellini et al., 2003; Kawai et al., 2004), and possibly metabolized (Reddy et al., 2009). When the molecular weight distribution at about 5.0 kDa or less contributes to a substantial part, e.g., 20% of the polymers under 1.0 or 2.0 kDa, this fraction can be, by comparison, rapidly biodegraded due to the microbial contribution (Chiellini et al., 2003; Kawai et al., 2004). In this study, based on the changes in molecular weight fraction distribution (Fig. 2e and f, Table S3), depolymerization and biodegradation of polymers resulted in a decrease of polymer fractions with molecular weight greater than 1000 kDa with PE-1, thus indicating positive capacity of degrading large polymers in the larvae. The decrease of polymer fractions of less than 10.0 kDa in the frass was likely due to the more rapid rate of degradation of these small polymers relative to larger polymers; the accumulation of fractions with 10.0 to 100.0 kDa could be the results of polymer fragments generated from the depolymerization of larger polymers and relatively slower degradation rate of the middle sized polymers compared to small sized polymers.

The PDI is a measure of the broadness of the molecular weights of polymers (Bacon et al., 2016). After biodegradation, the PDI of PE-1 polymers dropped from 11.8 to 7.3 by *T. molitor*, and to 7.2 by *T. obscurus* while the PDI of PE-2 polymers dropped from 9.7 to 7.4 by *T. molitor* and to 6.8 by *T. obscurus* (Table 2), indicating that the PDI of the extracted polymers from frass of *T. molitor* and *T. obscurus* was significantly lower ($p < 0.05$) than the two LDPE foams (11.8 and 9.7). A decrease in PDI was reported during the biodegradation of biodegradable polymers by microbial cultures (Fukushima et al., 2011). In this study, the decrease in PDI was due to the decrease in both high-molecular weight polymers (>1000 kDa) and small sized polymers (<5.0 kDa), thus reducing the broadness of the molecular weights of polymers as described above.

3.3. Carbon mass balance of the ingested LDPE

The conversion of the carbon of LDPE to CO_2, larval biomass, and PE residue in frass was assessed by a carbon balance test over 18 days with 200 larvae. The results showed that the total carbon recovered in CO_2 released, residual LDPE in frass and increment of total biomass was nearly 80% or higher (Fig. 1e and f, Table S2). The other fraction (Fig. 1f) was likely attributed to biodegrade intermediates in frass as well as analytical deviations. The digestibility of digested PE-1 was 70.3 ± 3.6% and 72.3 ± 3.5% in the digestive system of *T. molitor* and *T. obscurus*, respectively and that of PE-2 was 63.4 ± 6.1% and 72.1 ± 3.9%. The carbon conversion ratio of the digested PE-1 to CO_2 (mg/mg) by *T. molitor* and *T. obscurus* larvae was 0.55 ± 0.20 and 0.41 ± 0.03 (Table S2) while that of PE-2 was 0.52 ± 0.03 and 0.50 ± 0.22, respectively (Table S2). At the end of test, only 2.37% and 1.96% carbon of ingested PE-1, and 1.61% and 2.5% of ingested PE-2 were assimilated to the biomass of *T. obscurus* and *T. molitor* larvae, respectively (Fig. 1f,

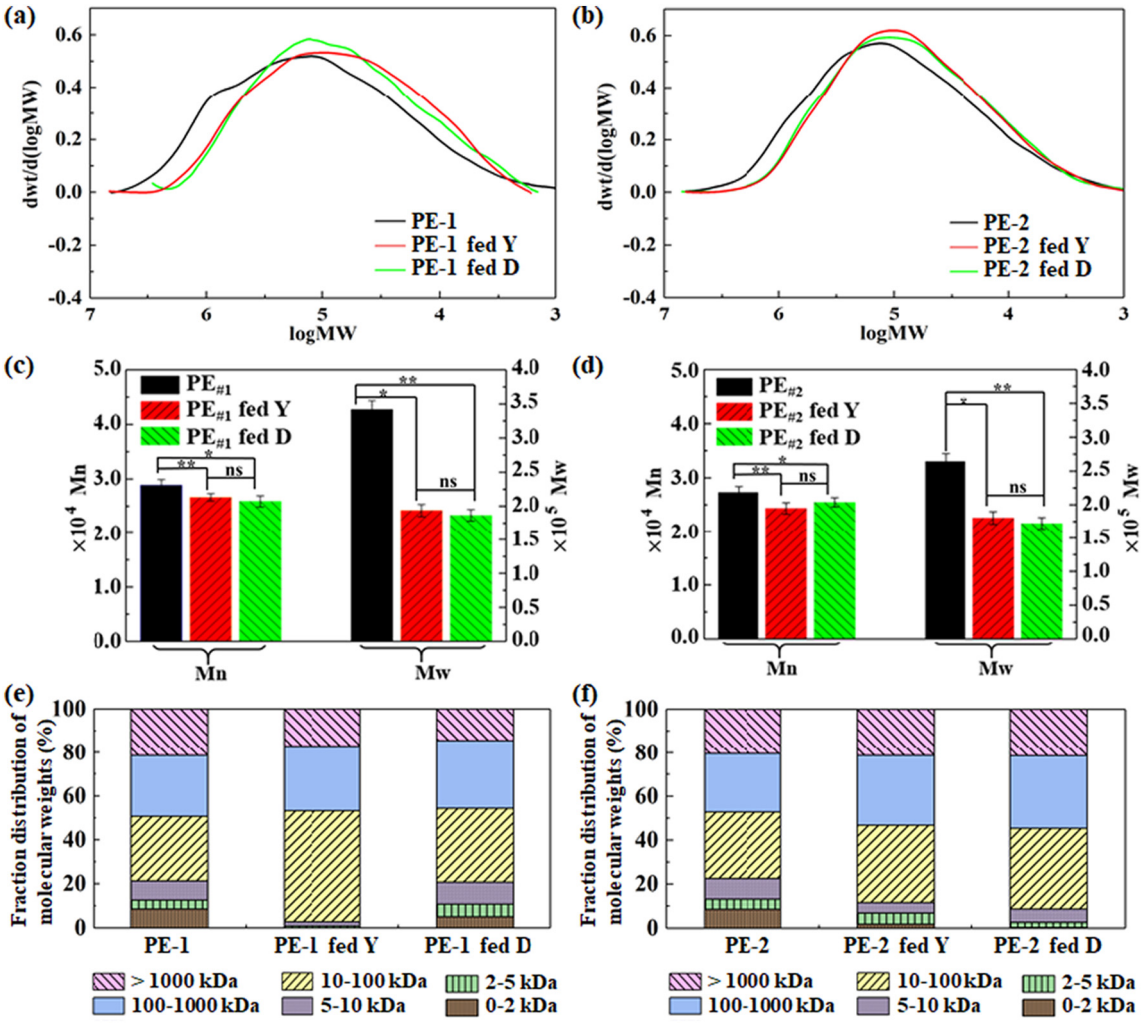

Fig. 2. Depolymerization and biodegradation of LDPE in *T. molitor* and *T. obscurus* larvae. Comparison of (a, b) molecular weight distribution (MWD) of LDPE feedstocks and frass of *T. molitor* and *T. obscurus* fed with respective PE-1 and PE-2. Comparison of (c, d) M_w and M_n of LDPE feedstocks and frass of *T. molitor* and *T. obscurus* fed with respective PE-1 and PE-2. (All values represent mean ± SD, n = 3. Significance (Student's *t*-tests) $p < 0.05$ indicated by *, $p < 0.01$ indicated by **, and no statistical significance indicated by ns.) (e, f) Fraction distribution of molecular weights of LDPE feedstocks and residual polymers in frass extracts from *T. molitor* and *T. obscurus* larvae fed respective PE-1 and PE-2 only.

Table S2). Around 50% carbon of ingested LDPE remained in frass and nearly 40% converted to CO_2 (Fig. 1f). Both *T. molitor* and *T. obscurus* performed similarly for carbon conversion (Fig. 1f). The results indicated that *T. obscurus* larvae did digest LDPE to CO_2 and assimilated a small fraction as biomass, which is consistent with the observation of LDPE conversion by *T. molitor* as reported by Brandon et al. (2018).

3.4. Evidence of biodegradation and oxidation of LDPE foams

Evidence of oxidation and depolymerization of the two LDPE foams was provided using FTIR analysis at the end of the test. FTIR spectra for the two LDPE foams and the corresponding residual LDPE polymers extracted from the frass samples of the larvae of *T. obscurus* and

Table 2
Molecular weight changes before and after digestion in *T. molitor* and *T. obscurus* larvae without and with antibiotic gentamicin treatment.

Sources	Mn, kDa	Mw, kDa	Mz, kDa	PDI	M_n reduction, %	M_w reduction, %	M_z reduction, %
PE-1 feedstock	28.9 ± 1.0	342.0 ± 13.0	1201.7 ± 24.0	11.8	–	–	–
Frass from PE-1 Y	26.7 ± 0.7	193.8 ± 9.1	661.2 ± 11.6	7.3	7.6 ± 0.9	43.3 ± 0.5	45.0 ± 0.5
Frass from PE-1 D	25.9 ± 1.0	186.8 ± 8.5	600.5 ± 12.0	7.2	10.4 ± 0.3	45.4 ± 0.4	50.1 ± 0.5
Frass from PE-1 Y (+G)	27.5 ± 1.3	299.9 ± 14.1	1035.0 ± 25.9	10.9	4.8 ± 0.4	12.3 ± 2.0	13.9 ± 0.3
Frass from PE-1 D (+G)	26.9 ± 1.4	285.9 ± 11.9	983.7 ± 24.6	10.6	6.9 ± 0.3	16.4 ± 1.9	18.1 ± 0.2
PE-2 feedstock	27.3 ± 1.1	264.1 ± 11.9	1022.9 ± 22.5	9.7	–	–	–
Frass from PE-2 Y	24.3 ± 1.1	180.3 ± 9.4	642.8 ± 11.6	7.4	11.0 ± 0.2	31.7 ± 0.5	37.2 ± 0.5
Frass from PE-2 D	25.5 ± 0.9	172.2 ± 8.5	552.6 ± 7.7	6.8	6.6 ± 0.7	34.8 ± 0.3	46.0 ± 0.4
Frass from PE-2 Y (+G)	25.8 ± 1.4	242.8 ± 15.4	929.0 ± 27.9	9.4	5.5 ± 0.2	8.1 ± 0.3	9.2 ± 0.4
Frass from PE-2 D (+G)	26.5 ± 1.0	237.2 ± 11.8	885.7 ± 20.4	8.94	2.9 ± 0.2	10.2 ± 0.6	13.4 ± 0.5

Note: Y = *T. molitor*; D = *T. obscurus*; +G = plus antibiotic gentamicin. PDI = polymer diversified index.

T. molitor were similar and revealed the bond changes and the formation of new functional groups through the incorporation of oxygen (Fig. 3a and b). FTIR analysis confirmed the identity of the LDPE film materials with peaks at 2921 cm^{-1} and 2852 cm^{-1} as C—H stretch bonds, the classical signatures of PE (Bombelli et al., 2017; Peng et al., 2020a; Yang et al., 2021a), but the intensities were much weaker in all frass samples. The characteristic peak for LDPE samples around 1500 cm^{-1} which represented the C — H ring, had high intensity in the PE-1 and PE-2 feedstock but disappeared in all frass samples. Appearance of a peak around 1700 cm^{-1} (C═O stretch) in the treated sample, is a classical signature of the carbonyl bond. Furthermore, the formation of an absorbance peak around 3300 cm^{-1} in the FTIR spectra of all frass samples is associated with the hydrogen bond of hydroxyl groups and/or carboxylic acid groups, suggesting that the surface had a change from hydrophobic to more hydrophilic property (Yang et al., 2018a; Peng et al., 2019). The appearance and disappearance of the peaks were similar to previous observations during LDPE biodegradation by *T. molitor*, *Z. atratus* and *G. mellonella* larvae (Bombelli et al., 2017; Brandon et al., 2018; Peng et al., 2020a; Yang et al., 2021a). The FTIR results indicated that *T. obscurus* larvae performed oxidation and biodegradation of LDPE in a same pattern as observed with *T. molitor* larvae.

Proton nuclear magnetic resonance (^{1}H NMR) spectra of the extracted polymers from the frass egested by *T. molitor* and *T. obscurus* larvae were compared with the spectra of raw LDPE foams (Fig. 3c and d). New peaks were observed in frass samples in the range of 1.0–2.0 ppm denoting a ^{1}H chemical shift and formation of CH_3 moiety, which is induced by the breakdown of long chain polymers after biodegradation (Skariyachan et al., 2018). Sharp new peaks in frass samples were observed in regions between 2.9 and 3.9 ppm, which are attributed to the CH_2 groups next to a nitrogen atom and the carbonyl group of the linker (Peng et al., 2020a). Also, new peaks at 2.78 ppm observed in frass samples corresponded to different metabolites or residues of proteins, like amino acids (Zainal et al., 2019). Furthermore, obvious increases in the intensities of regions at around 2.3 ppm in frass samples from LDPE-fed larvae also proved the existence of chemical modifications resulting in LDPE biodegradation as observed during HDPE biodegradation by lesser waxworms (*A. grisella*) and larvae of genus *Uloma* (Kundungal et al., 2019, 2021). The spectra of the frass sample indicated the presence of new peaks between 3.5 and 4.0 ppm (aldehyde moiety) which were absent in the original LDPE foams. Based on the shift in the peaks, the organic species possible were considered as: aliphatic protons from 0.77 ppm to 3.6 ppm region (H — C, CH_3, and CH_2); a shift to α position carbon atoms to Cα-C═O or C═N group (carbonyl/aminoic), and Cα — C — H groups (unsaturated allylic) (Kundungal et al., 2019), indicating the incorporation of oxygen, which was consistent with FTIR analyses (Fig. 3a and b). New peaks around $\delta_H = 5.36$ ppm in a region associated with alkene bonds (C═C — H) (Fig. 3c and d) were found in the FTIR spectra of the residual

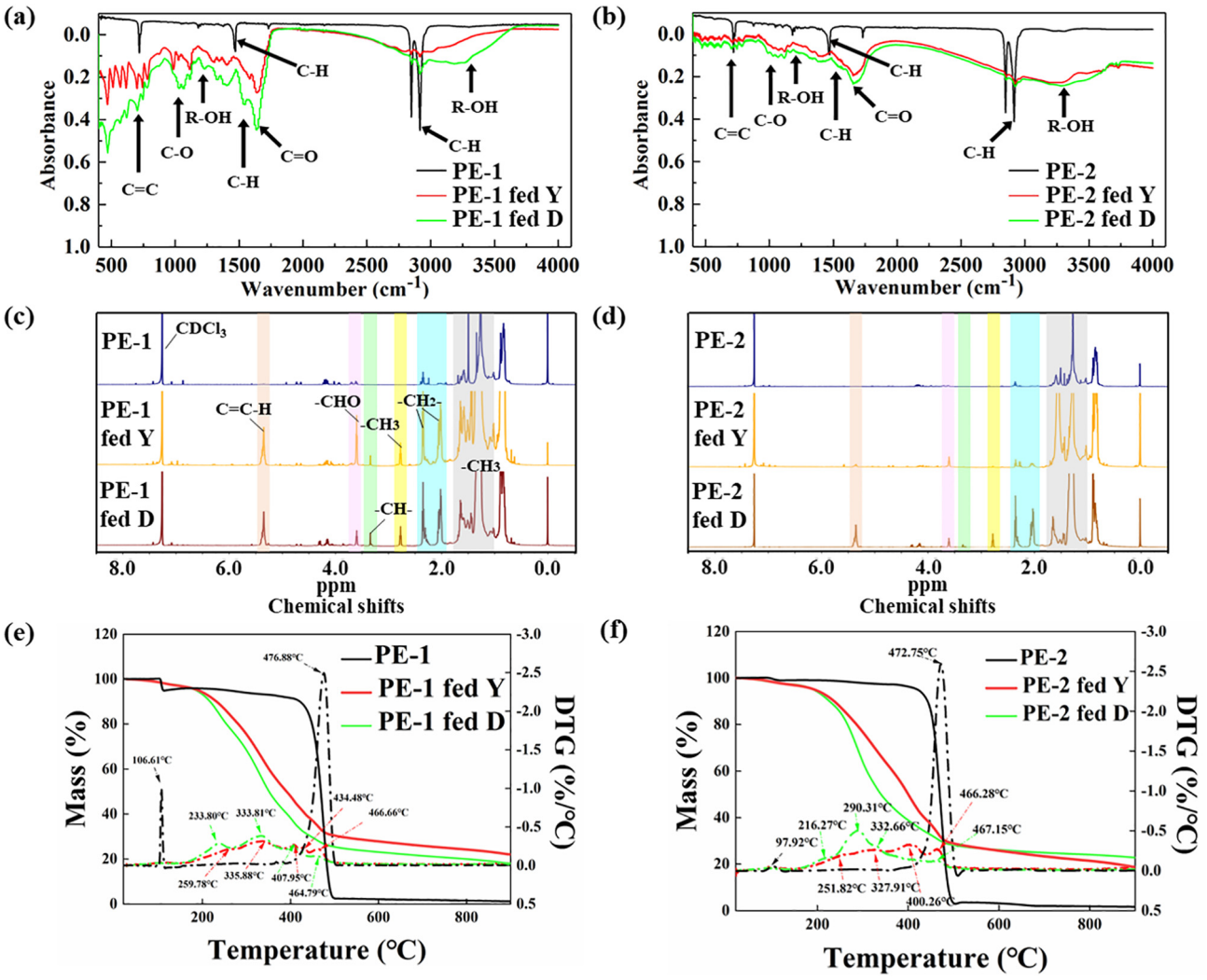

Fig. 3. Depolymerization and biodegradation of LDPE in *T. molitor* and *T. obscurus* larvae. Comparison of (a, b) FTIR spectra of LDPE feedstocks and the frass of *T. molitor* and *T. obscurus* fed respective PE-1 and PE-2 only. (c, d) ^{1}H NMR spectra of PE feedstocks and the extracts of the frass of *T. molitor* and *T. obscurus* fed respective PE-1 and PE-2 only. (e, f) TGA of PE feedstocks and the extracts of the frass of *T. molitor* and *T. obscurus* fed respective PE-1 and PE-2 only. Frass samples were obtained on day 36. Y = *T. molitor* larvae; D = *T. obscurus* larvae.

polymers from the frass samples, compared with the spectra of original LDPE foams. The results of ^{1}H NMR analyses supported oxidization and biodegradation of the two LDPE foams in the gut of *T. obscurus* larvae, similar to *T. molitor* larvae.

Thermal modifications of frass samples from PE-fed *T. molitor* larvae and *T. obscurus* larvae were detected and compared using TGA after the 36-day test. As shown in Fig. 3e and f, the TGA indicated an obvious mass loss in the raw LDPE that occurred from 412.03 to 500.55 °C where about 88.54% of weight loss occurred during only one stage and the maximum decomposition rate occurred at 476.88 °C. Five maximum decomposition rates appeared at 259.78, 335.88, 407.95, 434.48, and 466.66 °C for frass from *T. molitor* fed PE-1 only. Accordingly, four maximum decomposition rates appeared at 233.80, 333.81, 407.95, and 464.79 °C for frass from PE-1 fed *T. obscurus* (Fig. 3e). The decomposed parts in the ranges of 100–370 °C might be attributed to other biological digestive products (Yang et al., 2021b) including cannibalism and LDPE biodegradation residues from the guts of both species. Additionally, the mass loss ratios of the frass from *T. molitor* and *T. obscurus* larvae from 412.03 to 500.55 °C were respectively 14.41% and 9.77%, which were much lower than that of the PE-1 (88.54%), indicating that the frass contained not only PE but also more new biodegraded digestive products. This result confirmed the depolymerization of LDPE polymer as it passed through the digestive tract of the larvae. Similar observations were also found in the TGA tests using PE-2 foam and the frass of *T. molitor* and *T. obscurus* larvae (Fig. 3f). Different maximum decomposition rates observed from TGA analyses further suggested that different degradation pathways of LDPE polymers might be present in the two different species. Further studies are needed to characterize the metabolic pathways.

3.5. Antibiotics suppression test

The role of larval gut microbes in the biodegradation of LDPE was examined using the antibiotic gentamicin test. Previous inhibition halo tests with ampicillin, chloramphenicol, erythromycin, gentamicin, tetracycline, and vancomycin showed gentamicin had best ability to inhibit gut microbes of *T. molitor* larvae (Yang et al., 2015b). Prior to the test, we conducted an inhibition halo test with a group of six antibiotics (gentamicin, chloramphenicol, erythromycin, kanamycin sulfate, penicillin, and tetracycline), which were different from those tested by Yang et al. (2015b), to examine the ability to inhibit gut bacteria from *T. molitor* larvae. The results demonstrated that the sequence of inhibition halos (mm) was: gentamicin (32.08 ± 1.60) > penicillin (27.92 ± 1.98) > kanamycin sulfate (26.25 ± 2.85) > chloramphenicol (25.00 ± 1.36) > tetracycline (21.67 ± 1.636) (Fig. S2). Gentamicin showed the best ability to inhibit the growth of gut bacteria, with clearer and broader halos (Fig. S2).

Gentamicin sulfate and test procedures were similar to previous studies on the biodegradation of PS and PVC using *T. molitor*, *T. obscurus* and *Z. atratus* larvae (Yang et al., 2015b; Yang et al., 2018a; Peng et al., 2019; Peng et al., 2020a, b; Yang et al., 2021b; Wu and Criddle, 2021). After the larvae of *T. molitor* and *T. obscurus* were fed with the mixture of bran plus gentamicin, larval samples (20 per test group) were taken on day 7, 15, and 20 to prepare gut suspensions for the counting of gut microorganisms. The larvae not fed gentamicin served as samples for day 0 (or control). Gut bacterial numbers in *T. molitor* and *T. obscurus* larvae dropped significantly from 3.13×10^6 and 1.03×10^6 CFU per gut on day 0 to 1.11×10^5 and 0.99×10^5 CFU per gut on day 7, and continuously decreased to 6.2×10^4 and 4.4×10^4 CFU per gut on day 15. After antibiotics suppression for 15 days, the numbers of CFU decreased by almost two to three magnitudes (Fig. 4a and b), implying that the gut bacteria were effectively suppressed by gentamicin sulfate. The frass of the larvae fed on PE-1 and PE-2 was collected to analyze residual polymers extracted with 1,2,4-TCB using HT-GPC (Fig. 4c and d). The results showed that the depolymerization was negatively impacted but didn't stop in both *T. molitor* and *T. obscurus* larvae even when gut bacteria were severely inhibited (Fig. 4c and d). In the presence of gentamicin, *T. molitor* larvae reduced M_n of residual PE-1 and PE-2 by 4.8 ± 0.4% and 5.5 ± 0.2%, respectively; M_w of residual PE-1 and PE-2 by 12.3 ± 0.2% and 8.1 ± 0.3%, respectively; and M_z of residual PE-1 and PE-2 by 13.9 ± 0.3% and 9.2 ± 0.4%, respectively. Under similar conditions, *T. obscurus* larvae reduced M_n of residual PE-1 and PE-2 by 6.9 ± 0.3% and 2.9 ± 0.2%, respectively, M_w of residual PE-1 and PE-2 by 16.4 ± 1.9% and 10.2 ± 0.6%, respectively, and M_z of residual PE-1 and PE-2 by 18.1 ± 0.2% and 13.4 ± 0.5%, respectively. For both *Tenebrio* species, M_n of the residual polymers after antibiotics suppression was less significantly impacted with a decrease of only around 5%, i.e., no statistical difference between LDPE foams and residual LDPE polymers extracted from the frass of gentamicin suppression groups; the M_w and M_z of residual LDPE foams extracted from the frass of gentamicin suppression groups were significantly different ($p < 0.05$) from LDPE foams, suggesting that the LDPE depolymerization or biodegradation by both *T. molitor* and *T. obscurus* larvae was gut-microbe independent. A previous study similarly observed that LDPE depolymerization was independent in *T. molitor* larvae (Yang et al., 2021a). On the other hand, significant differences ($p < 0.05$) were observed in M_n, M_w, and M_z values between residual PE polymers extracted from the frass samples of the control and gentamicin treatments. This is evidence that gentamicin suppressed gut microbiota and also partially inhibited LDPE depolymerization by both *T. molitor* and *T. obscurus* larvae.

We also compared the changes in the fraction of small sized polymers (<2.0 kDa) in the frass of the larvae with and without antibiotic treatments. With antibiotic treatment, the frass samples from *T. molitor* and *T. obscurus* fed with PE-1 contained 3.89% and 5.98% of the small sized polymer (<2 kDa) and those fed with PE-2 included 4.54% and 3.23% of the small sized polymers, while the PE-1 form had 8.52% and PE-2 had 8.50% (Fig. 4e and f, Table S3), indicating the removal of small sized polymers under antibiotic suppression. However, the frass of the *T.molitor* and *T.obscurus* larvae of the control group (without antibiotic treatment) contained only <0.2% and 4.99% of the small sized polymers with PE-1, and 1.85% and 0.29% with PE-2. Similar to frass analyses without antibiotics, the fraction of polymers with molecular weight between 10.0 and 100 kDa were slightly increased or decreased in the frass samples with antibiotics (Fig. 4e and f, Table S3). However, depolymerization of larger polymers (>100 kDa) showed a reduced rate in the presence of antibiotics for PE-1 and basically unchanged for PE-2 suggesting that the synergetic efforts of gut microbes and digestive enzymes were depressed under antibiotic gentamicin conditions. The data in this study indicated that depolymerization of LDPE by both *T. molitor* and *T. obscurus* larvae was not completely dependent on gut microbes (Fig. 4a and b). This is different from the previously studies with PS that the PS depolymerization in *T. molitor* larvae and *T. obscurus* was inhibited or stopped in with antibiotic gentamicin (Yang et al., 2015a; Yang et al., 2018a; Peng et al., 2019; Yang et al., 2021a). Therefore, LDPE depolymerization in both *T. molitor* and *T. obscurus* larvae is likely gut microbe independent. On the other hand, the depolymerization of LDPE may be partially contributed to their intestinal digestive system (e.g., enzymatic, physical, and chemical reactions). In addition, the depolymerization patterns observed in this study was different from those reported by Yang et al. (2021b) who reported an increase in M_n and little change in M_w. This could be due to the differences in LDPE tested (different molecular weight distribution, polymer structure, crystallinity degree, etc.), and larval sources. Further study is needed to verify the enzyme(s) involved and the impact of LDPE materials and larval sources.

The results demonstrated that the larvae of *T. molitor* and *T. obscurus* are capable of depolymerizing LDPE, independent of gut microbes; and the biodegradation of LDPE in *T. molitor* and *T. obscurus* larvae is performed and accelerated by the synergistic driving forces from the larval digestive system and gut microbial activities. Future research is needed for in-depth investigations on PE-degrading mechanisms, including PE

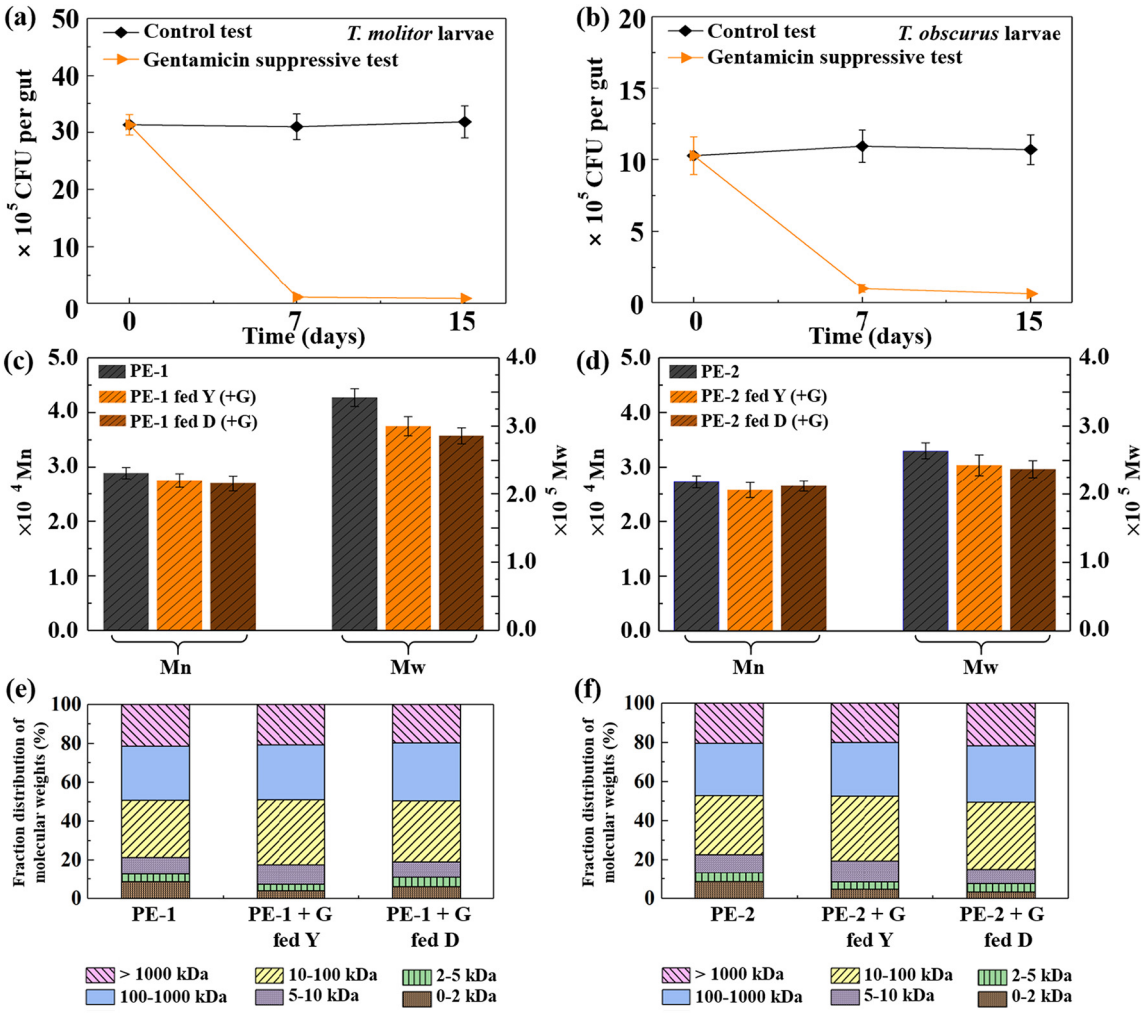

Fig. 4. Impact of antibiotic suppression on LDPE depolymerization in *T. obscurus* and *T. molitor* larvae. (a, b) Decrease in gut bacterial counts within *T. molitor* and *T. obscurus* larvae after feeding of gentamycin over a 7-day period. Bacteria counting test was conducted in duplicate. (c, d) Changes in M_n and M_w values of residual polymers from the frass of *T. molitor* and *T. obscurus* larvae receiving gentamycin compared to two PE foam feedstocks. (e, f) Fraction distribution of molecular weights of LDPE feedstocks and the residual polymers in frass from *T. molitor* and *T. obscurus* larvae fed PE-1 + G and PE-2 + G, respectively. Y = *T. molitor* larvae; D = *T. obscurus* larvae; +G = Gentamicin suppressive test.

types, MW distribution, structural arrangement, and etc. between different *Tenebrio* species. Additionally, the role of surviving gut microbes after antibiotic inhibition, although reduced in number, should also be further examined.

3.6. Gut microbiome analysis

Illumina sequencing of 16S rRNA genes was used to investigate the changes in the larval gut microbiome of both the species after LDPE foams were fed as the sole larval diet (Fig. 5). The analyses of microbial diversities across all eight experimental groups (Table 3) showed a total of 322,341 sequences, with an average length of 424.85 bps, and sampling coverage above 0.99, suggesting that Illumina Miseq sequencing was capable of detecting most of the reads (Lemos et al., 2011; Peng et al., 2019). The OTUs of LDPE-fed larvae groups were relatively lower than of those fed with bran as well as those of the unfed groups (Table 3), indicating that the LDPE-fed larval gut microbiome was selective towards to a lower community diversity.

The Shannon and Simpson indices indicated that species richness decreased substantially in the LDPE fed *T. molitor* groups. The gut microbiome from the bran-fed *T. molitor* larvae exhibited much higher community diversity than from bran-fed *T. obscurus* larvae based on the alpha diversity of the microbes measured via the Shannon and Simpson indices (Fig. S5). The number of OTUs of bran-fed *T. obscurus* of (113) was lower than that of bran-fed *T. molitor* (893), further indicating that the gut microbiome had higher diversity in *T. molitor* in comparison with *T. obscurus*.

A principal component analysis (PCA) and heatmap based on weighted UniFrac distances revealed clusters associated with different diets, showing clear clusters for LDPE-fed, bran-fed and unfed larvae (Fig. 5a and b). The microbiome of the *T. molitor* larvae fed with PE-1 and PE-2 was similar, as was the microbiome of the larvae from bran-fed and unfed groups. The similar clustering was also observed in different *T. obscurus* groups (Fig. 5a and b). Similar clustering of the microbiome of the larvae from bran-fed and unfed groups observed could be induced by the feed source of *T. molitor* and *T. obscurus* larvae. For the unfed (starvation) group, the larvae resorted to cannibalism for a food source. The composition of the larval biomass (proteins, fat, fiber, trace element etc.) may select for or maintain a gut community more similar to those fed bran LDPE because that LDPE contains only hydrocarbons resulting in selection of different communities. The alpha and beta diversity analysis suggested that the major gut microbiome of the larvae fed on LDPE foams did not differ dramatically with the two different LDPE foams diet used, while the composition of the microbial communities was distinctive between the different LDPE diets in the different *Tenebrio* species.

Differential abundance analysis of the gut microbiome of *T. molitor* and *T. obscurus* was used to assess whether particular OTUs were

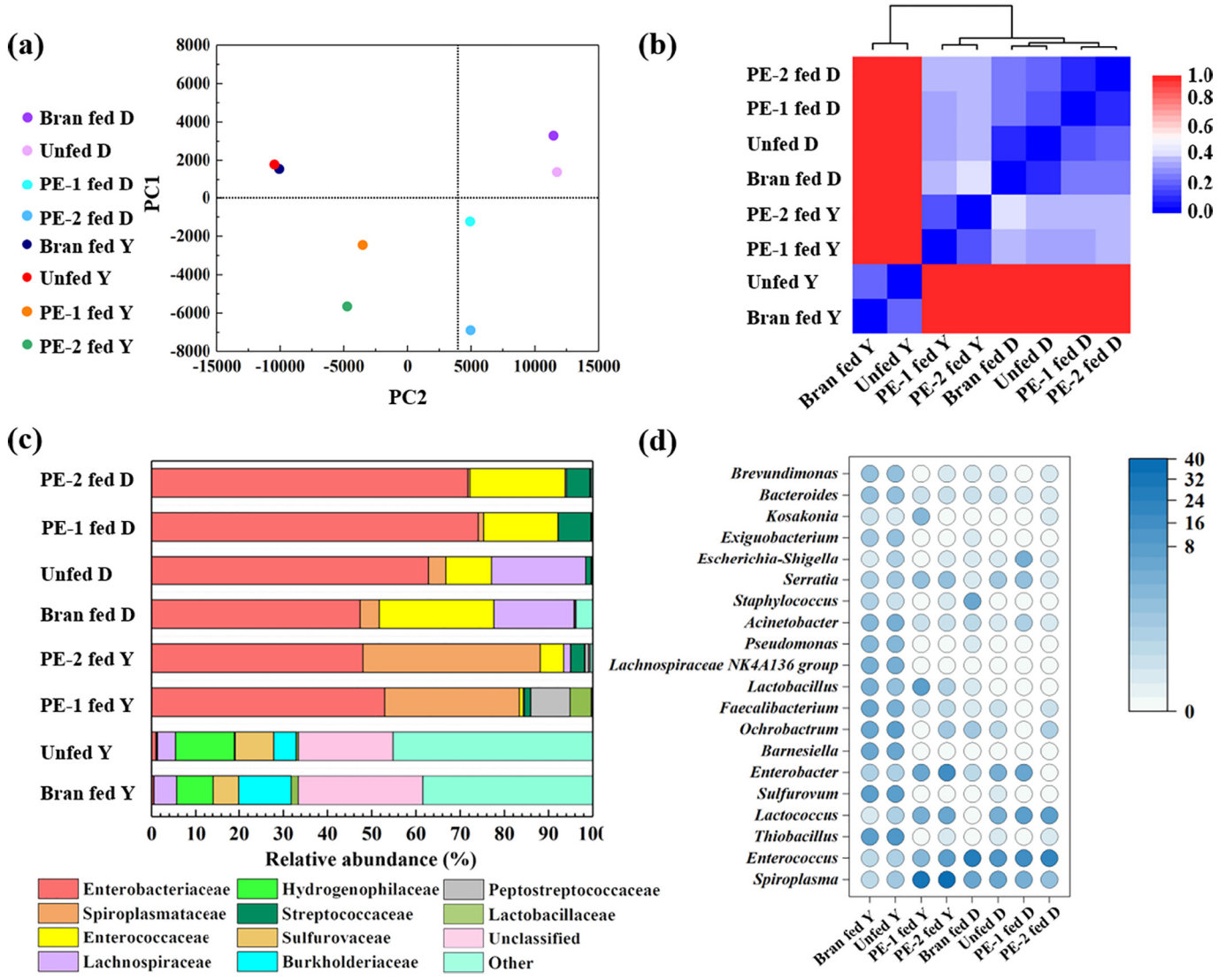

Fig. 5. Gut microbial community analysis of *T. molitor* versus *T. obscurus* larvae fed with PE-1 and PE-2 only, bran diet versus unfed controls using samples on day 36. (a) Principal component analysis based on weighted-UniFrac. (b) Community diversity distance analysis based on weighted-UniFrac. (c) Relative bacterial abundances of dominant populations (top 10) in the gut microbiomes of *T. obscurus* and *T. molitor* larvae fed the different diets at the family level. (d) Relative abundances at the genus levels. The analysis was based on the top 20 abundant genera in the eight samples. The color intensity of the scale indicates the relative abundance. Y = *T. molitor* larvae; D = *T. obscurus* larvae.

associated with diet shifts. Based on relative abundance, the gut microbiome was markedly different between *T. molitor* larvae fed with LDPE and control (bran-fed and unfed) larvae groups (Fig. 5c). Enterobacteriaceae and Spiroplasmataceae were the predominant families in the gut of *T. molitor* larvae fed with LDPE (Fig. 5c). The ingestion of LDPE resulted in the increase in relative abundance of Enterobacteriaceae from 0.41% to 52.91% and 48.01% in the groups fed on PE-1 and PE-2, respectively. Family Enterobacteriaceae contains LDPE-degrading *Enterobacter absuriae* YT1 which were isolated from the guts of *Plodia interpunctella* larvae previously (Yang et al., 2014). Increase in the Enterobacteriaceae family was also reported in the gut of *T. molitor* larvae from various sources (Yang et al., 2018b) and in

Table 3
Bacterial Diversity analyses based on illumine sequencing of the 16S rRNA Gene amplicons by two tested *Tenebrio* genera.

Samples	OTUs	Chao 1	Observed	Shannon	Simpson
Bran fed Y	893 ± 4.16	963.59 ± 2.17	851.50 ± 1.42	7.63 ± 0.29	0.98 ± 0.04
Unfed Y	684 ± 5.77	772.54 ± 3.07	634.50 ± 0.95	7.11 ± 0.09	0.97 ± 0.01
PE-1 fed Y	54 ± 3.21	88.50 ± 1.07	54.00 ± 0.10	2.70 ± 0.33	0.80 ± 0.04
PE-2 fed Y	94 ± 2.52	121.79 ± 1.33	83.10 ± 0.91	2.59 ± 0.31	0.77 ± 0.05
Bran fed D	113 ± 3.61	160.33 ± 0.59	89.90 ± 0.10	1.99 ± 0.12	0.68 ± 0.03
Unfed D	89 ± 2.08	136.06 ± 1.73	67.50 ± 1.29	2.17 ± 0.13	0.70 ± 0.02
PE-1 fed D	61 ± 2.00	121.91 ± 1.68	57.50 ± 1.74	2.27 ± 0.13	0.69 ± 0.01
PE-2 fed D	95 ± 3.05	123.38 ± 1.17	74.40 ± 1.92	1.97 ± 0.20	0.70 ± 0.01

Note: The initial number: Y = *T. molitor* larvae, 500; D = *T. obscurus* larvae, 500.

snails- *Achatina fulica,* (Song et al., 2020) during PS degradation. The relative abundance of Spiroplasmataceae significantly increased from 0.04% to 30.44% and 40.08% in PE-1 and PE-2-fed *T. molitor* larvae, respectively. Observation of Spiroplasmataceae was reported in *T. molitor* larvae which received bran, LDPE, PS and bran plus LDPE or PS (Brandon et al., 2018), and PLA as well as PLA plus bran (Peng et al., 2021). However, the role of this family in LDPE or PS degradation was not clear; the family contains a single genus, *Spiroplasma*, whose members are regularly associated with arthropod or plant hosts. In this study, *Spiroplasma* sp. might be associated with biodegradation of intermediates of LDPE (Yang and Wu, 2020; Peng et al., 2021). The relative abundance of the Enterococcaceae, Streptococcaceae, and Lactobacillaceae families also changed noticeably in LDPE-fed groups, which were found as gut bacteria or plastic degradation-associated bacteria in *T. molitor* gut microbiome (Yang et al., 2018b; Peng et al., 2019). The dominant families in the gut of *T. obscurus* fed with LDPE were Enterobacteriaceae, Enterococcaceae, and Streptococcaceae (Fig. 5c). Among the three dominant families, Enterobacteriaceae was the dominant population in the gut microbiome of larvae fed with PE-1 and PE-2, with 74.06% and 71.71% relative abundance, which was respectively increased by 26.73% and 24.38% compared with the larvae fed with bran only (relative abundance 47.32%). Enterobacteriaceae was slightly higher in the proportion of the LDPE fed *T. obscurus* larvae than those bran fed and unfed. Moreover, in the study by Peng et al. (2019), Enterobacteriaceae was one of the dominant families in PS fed *T. obscurus* and *T. molitor* larvae. Previous work by Brandon et al. (2018) found that some gut bacterial strains associated with LDPE and PS degradation by *T. molitor* belonged to the family Enterobacteriaceae. The results of this study indicated that microbiome capable of depolymerizing LDPE is widely present in *T. obscurus* larvae guts (Fig. 5c). Additionally, the families of Enterococcaceae and Streptococcaceae which are strongly associated with PS-degradation (Yang et al., 2018b; Peng et al., 2019) were observed in the gut microbial communities of both *Tenebrio* species, indicating that they were also significantly related to LDPE biodegradation in this study.

The differences in bacterial taxa between the larval guts of *Tenebrio* species fed with LDPE foams and bran or unfed groups at the genus level are presented in Fig. 5d. The predominant genus in the gut of LDPE-fed *T. molitor* larvae was affiliated with *Spiroplasma* sp. (relative abundance 30.44% for PE-1 and 40.08% for PE-2), whereas *Enterococcus* sp. (relative abundances of 16.82% and 21.50% for PE-1 and PE-2, respectively) dominated the gut microbiome of LDPE-fed *T. obscurus* larvae (Fig. 5d). Previous studies found that *Spiroplasma* sp. and *Enterococcus* sp., belong to families Spiroplasmataceae and Enterobacteriaceae, were strongly associated with LDPE and PS degradation in *T. molitor* (Brandon et al., 2018; Przemieniecki et al., 2019). *Lactococcus* sp., *Enterobacter* sp. and *Serratia* sp., which are associated with PE degradation (Yang et al., 2014; Brandon et al., 2018), shifted to higher relative abundances in LDPE fed larvae than those in bran-fed or unfed larvae (Fig. 5d), indicating that these genera were significantly related to LDPE-fed *T. molitor* larvae. We hypothesize that *Enterococcus* sp. was one of the gut microbes performing PE degradation in the *Tenebrio* genera. In this study, three previously suggested LDPE-degrading genera *Enterobacter* sp. and *Serratia* sp., and *Escherichia-shigella* (Peng et al., 2021) were not detected from the gut microbiome of PE-2 fed *T. obscurus* larvae. Additionally, *Kosakonia* sp., which was associated with LDPE and PS degradation in *T. molitor* (Brandon et al., 2018; Peng et al., 2019), was only detected in the gut microbiome of PE-1 fed *T. molitor* larvae. Abundances of *Bacillus* and *Acinetobacter*, which contain isolated LDPE-degrading strains from *P. interpunctella* (Yang et al., 2014) and *T. molitor* (Yin et al., 2020) were not significant or not increased in this study when LDPE was fed. This may be due to the source different in the gut microbiomes which did not include PE-degrading strains of *Bacillus* and *Acinetobacter*. The analyses using Illumina sequencing of the 16S rRNA gene demonstrated that the gut microbiome was shaped by different diets, resulting in a significant difference in larval guts community structures of the two *Tenebrio* genera.

4. Conclusions and future studies

The results of this study demonstrated the following conclusions:

a) Dark mealworms, the larvae of another member of darkling beetle, *Tenebrio obscurus* which belongs to the same genus of *Tenebrio molitor* (yellow mealworms) are also capable of biodegrading LDPE polymers.
b) The depolymerization of LDPE polymers is independent or less dependent on gut microbes, similar to *T. molitor*. The larvae of the two species were fed two different commercial LDPE foams with respective M_n of 28.9 and 27.3 kDa, and M_w of 342.0 and 264.1 kDa and digested both LDPEs.
c) Analysis of distribution of molecular weights of LDPE polymers indicated that low-molecular weight regions (especially <5.0 kDa) rapidly degraded, and large molecular weight regions (>1000 kDa) also decreased as the larvae performed broad LDPE depolymerization.
d) Carbon balance analysis indicated that majority of digested LDPE converted/oxidized to CO_2.
e) Biodegradation of ingested LDPE was confirmed through the formation of oxidized intermediates and chemical/physical modifications which was confirmed using FTIR, ^{1}H NMR and TGA analyses.
f) The low extent of broad depolymerization under antibiotic gentamicin suppression suggested the presence of digestive enzyme(s) to break down LDPE.
g) Gut community was changed significantly after the larvae received a diet of LDPE compared with those fed on bran and unfed.
h) Gut microbiome analyses revealed that the predominant gut microbes associated with LDPE degradation included bacterial families of Enterobacteriaceae, Enterococcaceae and Streptococcaceae. At the genus level, *Spiroplasma* sp. and *Enterococcus* sp. were strongly associated with LDPEs biodegradation.

Further studies will focus on mechanisms of plastic biodegradation in *T. obscurus* and other darkling beetles. Although the depolymerization of LDPE in both *T. obscurus* and *T. molitor* is independent of gut-microbe, the collaboration of the insect host and its microbiome could still play a critical role in the rapid biodegradation of plastics. A recent report by Brandon et al. (2021) indicated that *T. molitor* larvae secretes one or more emulsifying factor(s) with 30–100 kDa that mediate plastic bioavailability and significantly enhanced PS biodegradation by gut bacterial enrichment from the larval gut. Further studies are needed to explore whether *T. obscurus* larvae also secrete the same or similar emulsifying factor(s) and the efficacy of the factor(s) on the biodegradation of LDPE as well as other plastics (e.g., PVC, PP, PET, etc.) which have not been tested for biodegradation by *T. obscurus*.

CRediT authorship contribution statement

S. S. Yang: Conceptualization, Methodology, Supervision, Data validation, Writing – original draft, review & editing, Funding acquisition. **M.Q. Ding**: Methodology, Formal analysis, Investigation, Methodology, Writing - review & editing. **Z. R. Zhang**: Investigation, Sample collection & analysis. **S. W. Bai**: Conceptualization- experimental design. **G. L. Cao**: Conceptualization- experimental design. L. Zhao: Conceptualization-experimental design. **J. W. Pang**: Conceptualization- experimental design. **J. Ding**: Conceptualization- experimental design, Funding acquisition. **D. F. Xing**: Conceptualization- experimental design. **N. Q. Ren**: Conceptualization- experimental design. **W. M. Wu**: Conceptualization-experimental design, Methodology, Supervision, Data validation, Writing drafting, review & editing. All authors contributed to manuscript reviewing & editing.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.147915.

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