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Biodegradation of polypropylene by yellow mealworms (*Tenebrio molitor*) and superworms (*Zophobas atratus*) via gut-microbe-dependent depolymerization

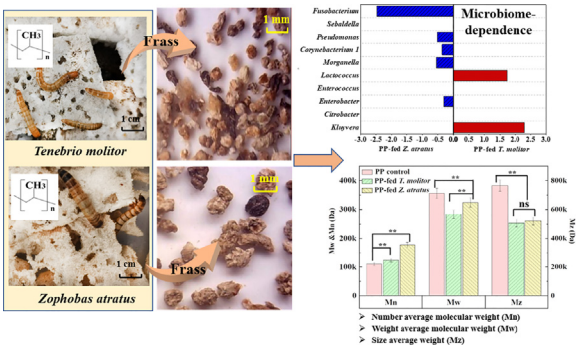
Shan-Shan Yang [a,*], Meng-Qi Ding [a], Lei He [a], Chun-Hong Zhang [a], Qing-Xiang Li [a], De-Feng Xing [a], Guang-Li Cao [a], Lei Zhao [a], Jie Ding [a], Nan-Qi Ren [a], Wei-Min Wu [b,*]

[a] State Key Laboratory of Urban Water Resource and Environment, School of Environment, Harbin Institute of Technology, Harbin 150090, China
[b] Department of Civil and Environmental Engineering, Department of Chemistry, William & Cloy Codiga Resource Recovery Center, Center for Sustainable Development & Global Competitiveness, Stanford University, Stanford, CA 94305, USA

HIGHLIGHTS

- *Tenebrio molitor* and *Zophobas atratus* consumed PP foam.
- PP was biodegraded via limited extent depolymerization with M_z reduction >32%.
- Antibiotic tests indicated gut-microbe dependent biodegradation in both larvae.
- Microbiome shifted with distinct dominant species during PP degradation.

GRAPHICAL ABSTRACT



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ABSTRACT

Polypropylene (PP), a fossil-based polyolefin plastics widely used worldwide, is non-hydrolyzable and resistant to biodegradation as a major source of plastic pollutants in environment. This study focused on feasibility of PP biodegradation in the larvae of two species of darkling beetles (Coleoptera: Tenebrionidae) i.e., yellow mealworms (*Tenebrio molitor*) and superworms (*Zophobas atratus*) using PP foam with number-, weight-, and size-average molecular weights (M_n, M_w, and M_z) of 109.8, 356.2, and 765.0 kDa, respectively. The tests were conducted in duplicates with respective larvae (300 *T. molitor* and 200 *Z. atratus* each incubator) at 25 °C and 65% humidity for over a 35-day period. The larvae of *T. molitor* and *Z. atratus* fed with PP foam as sole diet consumed PP at 1.0 ± 0.4 and 3.1 ± 0.4 mg 100 larvae^{-1} days^{-1}, respectively; when fed the PP foam plus wheat bran, the consumption rates were enhanced by 68.11% and 39.70%, respectively. Gel permeation chromatography analyses of the frass of *T. molitor* and *Z. atratus* larvae fed PP only indicated that M_w was decreased by 20.4 ± 0.8% and 9.0 ± 0.4%; M_n was increased by 12.1 ± 0.4% and 61.5 ± 2.5%; M_z was decreased by 33.8 ± 1.5% and 32.0 ± 1.1%, indicating limited extent depolymerization. Oxidation and biodegradation of PP was confirmed through analysis of the residual PP in frass. Depression of gut microbes with the antibiotic gentamicin inhibited PP depolymerization in both *T. molitor* and *Z. atratus* larvae. High throughput 16S rRNA sequencing revealed that *Citrobacter* sp. and *Enterobacter* sp. were associated with PP diets in the gut microbiome of *Z. atratus* larvae while *Kluyvera* was predominant in the *T. molitor* larvae. The results indicated that PP can be biodegraded in both *T. molitor* and *Z. atratus* larvae via gut microbe-dependent depolymerization with diversified microbiomes.

* Corresponding authors.
E-mail addresses: shanshanyang@hit.edu.cn (S.-S. Yang), billwu@stanford.edu (W.-M. Wu).

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1. Introduction

Plastic wastes have been an environmental concern for decades, with up to 6300 million metric tons of the wastes generated to date (Geyer et al., 2017). Among 359 million tons of global plastic production in 2018, polypropylene (PP) is one of six widely used plastic polymers, based on polymer demand in Europe, i.e. polyethylene (PE), 29.8%; polypropylene (PP), 19.3%; polyvinyl chloride (PVC), 10.2%; polyurethane (PUR), 7.7%; polyethylene terephthalate (PET), 7.4%; and polystyrene (PS), 6.6% (Plastics Europe, 2019; Rodrigues et al., 2019). Polypropylene (PP) is a rigid crystalline thermoplastic, belonging to the polyolefin family of polymers, and is one of the most widely used polymers present in everyday objects such as toys, bottles, containers, pipes, houseware, bags, trays, agricultural film, food packaging film, etc. (Raddadi and Fava, 2019). After PE, PP is the most profitable plastic with revenues expected to exceed US$145 billion by 2019 (Wikipedia, 2020). Global PP production was 55 million tons in Khoramnejadian, 2013 and has been projected to increase with sales growth at a rate of 5.8% per year until 2021 (Wikipedia, 2020), although this estimation may be revised due to recent COVID-19 pandemics. PP is a linear hydrocarbon resin with chemical formula $(C_3H_6)_n$. Upon polymerization, PP can form three basic chain structures depending on the position of the methyl groups: atactic (aPP) with irregular methyl group (CH_3) arrangement; isotactic (iPP) with CH_3 arranged on one side of the carbon chain; and syndiotactic (sPP) with alternating CH_3 arrangements (Natta and Corradini, 1960; Huang et al., 2020). Commercially available PP products usually have an isotactic index between 85 and 95% i.e., consisting of 89% iPP and small amount of aPP (Wikipedia, 2020). PP is also major source of plastic solid wastes (Achilias et al., 2007; He et al., 2019) and pollutants from microplastics (MPs) and nanoplastics (NPs) in various environments including marine, soil, landfill leachate, etc. (Scheurer and Bigalke, 2018; He et al., 2019; Wu et al., 2017; Wang et al., 2021), biota collected from the field (de Sá et al., 2018) as well as major MPs in traditional medicinal materials (Lu et al., 2020).

Research on the biodegradation of plastics in environment has been reported since 1970s. To date, most of the researchers have focused on the biodegradation of PE, PET and PS while PP biodegradation has limited publications (Arutchelvi et al., 2008; Gewert et al., 2015; Krueger et al., 2015; Raddadi and Fava, 2019). Like PE, PP polymer belongs to non-hydrolyzable plastics (Wei and Zimmermann, 2017) and consists of hydrocarbons with high hydrophobicity but differs by the presence of a methyl group on every subunit of the polymer backbone (Jeon and Kim, 2016; Krueger et al., 2015). Biodegradation of isoparaffin, which consists of side chains, is more difficult than that of linear n-paraffin (Atlas and Bartha, 1972). Therefore, PP, with methyl side chains on every repeating unit, is expected to undergo a more difficult biodegradation process than PE (Arkatkar et al., 2009; Jeon and Kim, 2016). During the biodegradation of plastics, the first step is depolymerization or breaking down polymer chains by oxidation or/and hydrolysis; the degraded products are further oxidized and mineralized. The depolymerization of plastics can be characterized using gel permeation chromatography (GPC) analysis which provides number average molecular weight (M_n), weight average molecular weight (M_w), size average weight (M_z), and molecular weight distribution (MWD), which are widely used as indicators to characterize depolymerization (Ohtake et al., 1998; Albertsson et al., 1998; Peng et al., 2020b; Yang et al., 2015a, 2018a, 2018b, 2021).

To date, reports on PP biodegradation by microorganisms remain limited. Several researchers are attempting to discover PP-degrading microbial cultures (bacteria, fungi etc.) from open storage yard soils, compost soil, waste management landfills, sewage treatment plants, ocean life, mangrove systems, and etc. (Supplementary information, Table S1). Several researches used two-step strategies to achieve biodegradation i.e., physicochemical pre-treatments followed by biological degradation by microorganisms (Gu et al., 1996; Arkatkar et al., 2009; Jeyakumar et al., 2013; Fontanella et al., 2013; Khoramnejadian, 2013; Sepperumal and Markandan, 2014; Aravinthan et al., 2016). Additional research suggested improving the biodegradability of PP materials using grafting with biodegradable polymers such as lignin or blending starch (Krueger et al., 2015). Cacciari et al. (1993) reported biodegradation of iPP in a selective enrichment culture community, containing *Pseudomonas chlororaphis*, *P. stutzert*, *Vibrio* sp., *Bacillus* sp. and sulfate reducing bacteria over 175 days with about 40% mass removal of iPP added (1000 mg), which has been the highest removal efficiency reported to date. Artham and Doble (2009) found only 0.65% weight loss of PP in sea water. Arkatkar et al. (2010) reported in vitro weight loss of up to 2.5% after one year in minimal medium when UV-pretreated PP films were incubated with *Bacullus* and *Pseudomonas* strains. However, PP without pretreatment showed little changes. According to previous studies (Wypych, 2008; Curtzwiler et al., 2019), the initial degradation mechanism of PP is believed to analogous to PE, i.e., oxygen absorption and attack of the polymer backbone followed by increased oxygenated functional groups, chain scission, and subsequent methyl ketones.

Since 1950s, researchers have observed that some larvae and adults of insects, especially belonging to darkling beetles (Coleoptera: Tenebrionidae) and pest moths (Lepidoptera: Pyralidae) chew, ingest and damage plastic package materials (Gerhardt and Lindgren, 1954; Cline, 1978; Newton, 1988). Widely plastic contamination has resulted in the ingestion and detention of plastic MPs in many invertebrates in environment e.g., recently, MPs in 20 types of invertebrates in terrestrial environments (Lu et al., 2020) and observation of MPs in earthworms (Lwanga et al., 2016, 2017) and snails (Song et al., 2019, 2020). The possibility of biodegradation of ingested plastics in the invertebrates become attractive. Among the invertebrates, yellow mealworms (the larvae of *Tenebrio molitor* Linnaeus 1758), superworms (larvae of *Zophobas atratus* Fabricius 1775) and dark mealworms (larva of *Tenebrio obscurus* Fabricius 1792), the members of darkling beetles belonging to Coleoptera: Tenebrionidae (Robinson, 2005), have been studied for biodegradation of PS foam (Yang et al., 2015a; Peng et al., 2020a, 2020b). This study was initiated by high school students for science fairs, and then investigated by academic researchers (Yang et al., 2015a; Yang et al., 2018a). *T. molitor* larvae are capable of ingesting and biodegrading no hydrolysable plastics rapidly within short gut detention time of 12–15 h, including PS (Brandon et al., 2018; Peng et al., 2019; Yang et al., 2015a, 2015b, 2018a, 2018b), LDPE (Brandon et al., 2018; Yang et al., 2021), and PVC (Peng et al., 2020b). Research on biodegradation of PS and LDPE with *Z. atratus* larvae was initiated ten years ago (Miao and Zhang, 2010) and their capacities of depolymerization and biodegradation of polystyrene foam (Yang et al., 2020; Peng et al., 2020a) and LDPE (Peng et al., 2020a) have been verified recently. Insects harbor a variety of microorganisms in the gut, providing their host with physiological and ecological advantages (Jang and Kikuchi, 2020). The roles of symbiotic bacteria colonizing the gut play an important role in the digestion of ingested food (Genta et al., 2006; Jang and Kikuchi, 2020) and plastics in plastic-degrading insets (Yang et al., 2014; Yang et al., 2015b). Depression of gut microbes using antibiotics is commonly used method to examine the role of gut microbes in digestion of ingested refractory organic food (Genta et al., 2006). Antibiotic treatment using gentamicin, ampicillin or mixed antibiotics can reduced gut bacterial community by more than two magnitudes (Jung et al., 2014; Yang et al., 2015b; Yang et al., 2018a; Yang et al., 2021). Feeding antibiotic gentamicin-containing diet resulted in inhibition of PS depolymerization in *T. molitor* larvae, indicating that PS biodegradation is involved with activities of gut microbes or gut microbe-dependent (Yang et al., 2015b, 2018a). However, depolymerization of LDPE in *T. molitor* larvae is less gut microbe dependent or independent (Yang et al., 2021). *T. molitor* larvae are also capable of ingesting (Bożek et al., 2017), biodegrading and partially mineralizing PVC polymers (Peng et al., 2020b), as well as biodegrading

vulcanized styrene-butadiene rubber and tire crumb (Aboelkheir et al., 2019).

The PS and LDPE degradation has also been studied with another genus of darkling beetles, superworms (larvae of *Zophobas atratus*, synonym as *Zophobas morio*) for consumption of PS, LDPE and PVC (Miao and Zhang, 2010; Peng et al., 2020a, 2020b; Yang et al., 2020). Recent results indicated that *Z. atratus* larvae are capable of performing gut-microbe-dependent PS depolymerization/degradation with broad or limited extent depolymerization (Yang et al., 2020; Peng et al., 2020a) and LDPE degradation with a limited depolymerization pattern (Peng et al., 2020a). However, to date, the feasibility of biodegradation of PP in either *T. molitor* or *Z. atratus* larvae has not been reported. It remains unknown whether the larvae of *Tenebrio molitor* and *Zophobas atratus* degrade PP and how the reactions are performed.

The aims of this study were to investigate the feasibility and mechanisms of biodegradation of PP in *T. molitor* and *Z. atratus* larvae, the role of gut microbes in PP digestion/biodegradation, and the response of gut microbiomes to PP diet. We tested both *Tenebrio molitor* and *Zophobas atratus* from two sources in China. The results demonstrated that larvae of both *T. molitor* and *Z. atratus* do have capacity of biodegradation of PP

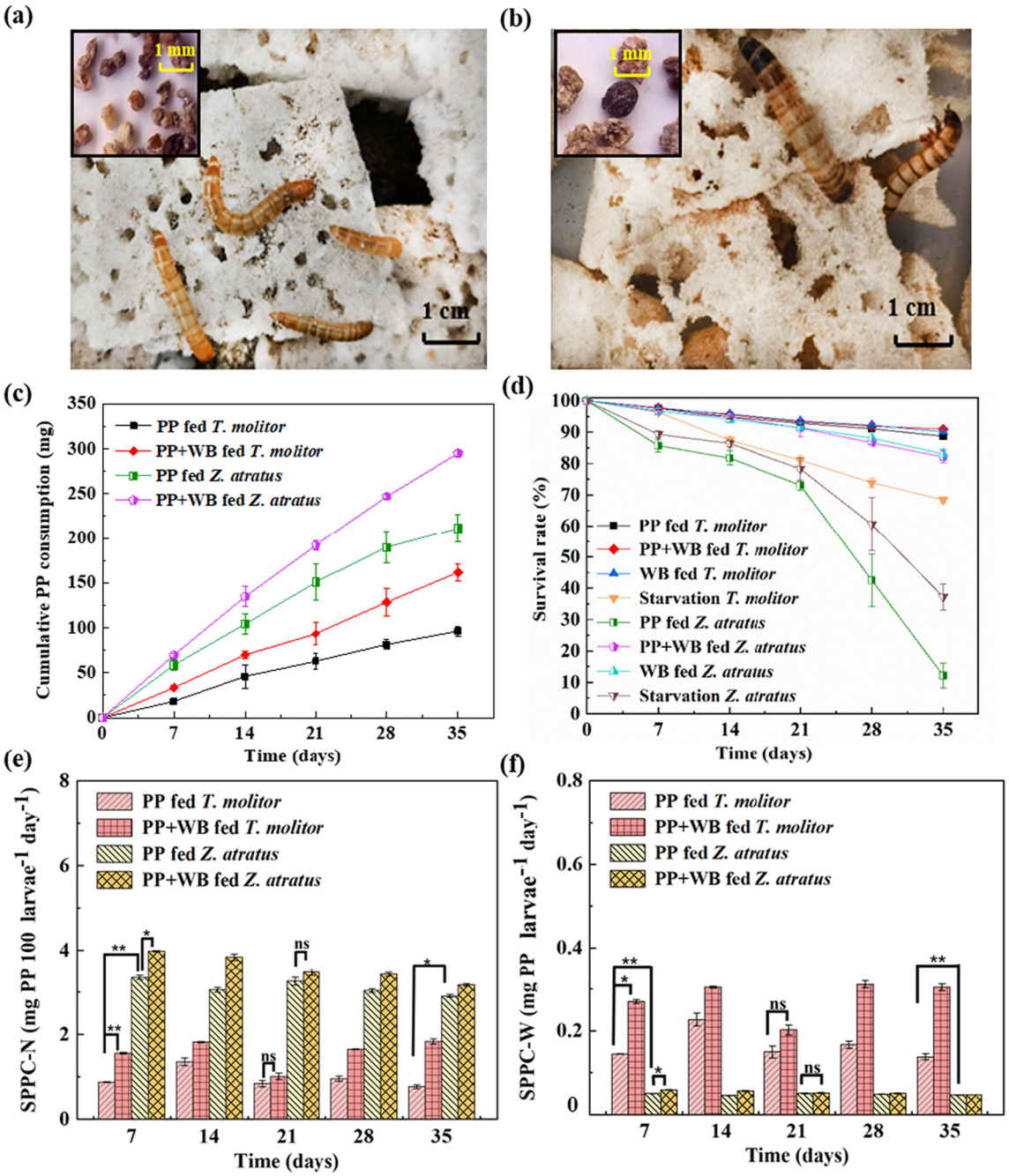

Fig. 1. Characterization of PP foam consumption by *T. molitor* and *Z. atratus* larvae. (a) PP foams-eating *T. molitor* larvae. (b) PP foams-eating *Z. atratus* larvae. (c) Comparison of PP consumption for *T. molitor* larvae and *Z. atratus* larvae fed with PP only and co-fed PP + WB. (d) Survival rates for *T. molitor* larvae and *Z. atratus* larvae fed with WB only, PP only, PP + WB co-diet, and unfed tests. (e) Specific PP consumption of 100 mg worms every day (SPPC-N, mg PP·100 larvae^{-1} d^{-1}) of both species fed with PP only and co-fed PP + WB. (f) Specific PP consumption of 1 g worms every day (SPPC-W, mg PP·g larvae^{-1} d^{-1}) of both the species fed with PP only and co-fed PP + WB over 35-day rearing period. (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.)

foams with limited extent depolymerization pattern, and the biodegradation is gut microbe dependent with significant shifts of gut microbiome during PP depolymerization and degradation.

2. Materials and methods

2.1. Sources of Tenebrio molitor and Zophobas atratus and test materials

Tenebrio molitor larvae (yellow mealworms) with a weight of 59.6 ± 2.2 mg/larva and a length of 2.0–2.5 cm were purchased from an insect farm named Pet food factory, Luoyang, Henan, China (Fig. 1a, Table 1). *Zophobas atratus* larvae (superworms) with 3.0–4.0 cm in length and weight of 611.0 ± 12.7 mg/larva were purchased from an insect breeding farm named Superworms House in Yangjiang, Guangdong, China (Fig. 1b, Table 1). The larvae of the two species were not fed with any antibiotics according to suppliers. Prior to experimentation, the larvae were fed with wheat bran (WB). The WB was purchased from Superworms House, Yangjiang, Guangdong, China. All WB feedstock fed on farm and in lab is natural without addition of any pesticides and antibiotics according to suppliers. All the larvae were stabilized to a 48-h starvation to empty their guts before experimentation.

PP foam (1.0 cm thickness), used as feedstock, was purchased from the online Foam dealer Kunshan balkline Company, Suzhou, China. HT-GPC analysis indicated that the PP foam had M_n of 356.2 kDa, M_w of 109.8 kDa, and M_z of 765.0 kDa (Table S3). The foam density was 0.02 g/cm^3 with a water angle of 131.5 ± 0.2 °C. Before the tests, the PP foams were washed with tap water and then distilled water, and subsequently dried at 30 °C for 2 days. Prior to feeding the larvae, the dried PP foams were put into an artificial climate chamber (25.0 ± 0.5 °C, humidity of 65 ± 5%) to reach stable weight under incubator condition.

2.2. Biodegradation PP foam

To assess PP consumption and biodegradation by the larvae, PP foam block (1.00 ± 0.05 g) was cut into irregular 5–6 cm cubes and cleaned with a stream of air. Each incubator received 300 *T. molitor* larvae or 250 *Z. atratus* larvae, respectively. The incubators were food grade polypropylene storage containers inoculated with about 2 larvae/cm^2 rearing density for *T. molitor* larvae and 0.5 larvae/cm^2 for *Z. atratus* larvae. During the rearing period, all the incubators were stored in a 250 L artificial climate chamber (Artificial climate incubator, Shanghai Shuli, Shanghai, China) controlled at 25.0 ± 0.5 °C with a humidity of 65 ± 5%, based on the optimal rearing conditions for biodegradation of PS by *T. molitor* larvae previous reported (Yang et al., 2018a).

Eight treatments were prepared based on feeding conditions: i) unfed *T. molitor*, ii) WB-fed *T. molitor*, iii) PP-fed *T. molitor*, iv) PP plus WB (PP + WB) fed *T. molitor*, (v) unfed *Z. atratus*, (vi) WB-fed *Z. atratus*, (vii) PP-fed *Z. atratus*, and (viii) PP + WB fed *Z. atratus*, respectively. Co-diet (WB) treatments were initially supplied with PP (1.0 g) plus WB (1.0 g) for both *T. molitor* and *Z. atratus*. Afterwards, an additional 1.0 g of bran was supplemented every 4 days. PP consumption was determined based on mass loss over incubation time by weighing residual PP foam weekly. Survival rates (SRs) of *T. molitor* and *Z. atratus* larvae were determined by counting the number of living larvae weekly and the test was ended on day 35. Dead bodies of larvae were removed immediately to reduce the mass consumed by cannibalism. Death rate was calculated as 100-SR (%). The cannibal rate was calculated on the basis of the difference between number of living larvae and dead bodies divided by the number of initial number of larvae inoculated. The specific PP consumption rates (SPPCR) were calculated based on the mass of PP consumed per 100 larvae per day (mg PP·100 larvae^{-1} d^{-1}) and the mass of PP consumed per larval weight per day (mg PP·g larvae^{-1} d^{-1}). The rates were measured on day 7, 14, 21, 28 and 35. At the end of the 35-day test, the larvae were cleansed of residual PP debris with compressed air, then transferred to a clean box for 12–18 h of frass collection. Frass samples were stored at 4 °C for further analysis. For DNA analyses, larvae samples (at least 30) were collected, preserved in high purity (99.999%) ethanol and stored at −80 °C. All tests were carried out in triplicates.

2.3. Antibiotic suppression test

An antibiotic suppression test was performed to examine the role of gut microbes on PP depolymerization/biodegradation in *T. molitor* and *Z. atratus* larvae with gentamicin, using established procedures described previously (Yang et al., 2015a; Yang et al., 2018a; Peng et al., 2019; Yang et al., 2021). Gentamicin suppressive groups (250 larvae of *T. molitor* or *Z. atratus* in each group) were fed antibiotic-treated bran containing gentamicin sulfate (30 mg/g bran) for 14 days and then supplied with PP foam for 7 days. Subsequently, the larvae for each species was randomly chosen from respective incubators on day 0, 7, and 14 to count the number of gut bacteria. To anatomize the guts of larvae, the larvae were decontaminated by 80% ethanol and rinsed using Milli Q water. The gut microbial levels during antibiotic treatment was monitored using a colony counting method with tryptic soy agar (TSA) medium as described in literatures (Kong et al., 2019; Peng et al., 2019). Gut contents were extracted and suspended in 5 mL sterile saline water, serial diluted from 10^{-1} to 10^{-7}, and cultivated on nonselective TSA plates at 37 °C for 24 h. After bacterial number counting on day 14, the remaining antibiotic-treated larvae were subsequently fed with PP foam. In order to maintain continuous suppression of the gut microbes, PP foam was fed alternatively with the antibiotic-treated WB diet. The frass and guts tissues from the antibiotic treatment groups and control groups were collected for further analysis.

Table 1
Characteristics of consumption of different diets (WB only, PP only, PP + WB, and unfed groups) by *T. molitor* and *Z. atratus* larvae.

Larval species	Feedstock	Initial weight, mg larva^{-1}	Cumulative PP consumed, mg	Survival rate, %	Cannibal rate[a], %	Death rate, %	Specific PP consumption rate[b]	
							SPPC-N, mg PP·100 larvae^{-1} d^{-1}	SPPC-W, mg PP·g larva^{-1} d^{-1}
Tenebrio molitor	Unfed	59.7 ± 1.3	nd	68.4 ± 1.0	11.0 ± 1.3	31.6 ± 0.8	nd	nd
	Bran	59.3 ± 0.3	nd	90.1 ± 0.4	1.7 ± 0.3	9.1 ± 0.4	nd	nd
	PP	60.6 ± 4.4	96.1 ± 5.3	88.7 ± 0.7	5.3 ± 0.7	11.3 ± 0.8	1.0 ± 0.4	0.2 ± 0.1
	PP + WB	59.0 ± 2.7	161.6 ± 9.2	91.0 ± 0.3	2.3 ± 0.7	9.0 ± 0.8	1.6 ± 0.3	0.3 ± 0.0
Zophobas atratus	Unfed	677.4 ± 12.2	nd	37.2 ± 4.2	59.3 ± 3.3	62.8 ± 1.2	nd	nd
	Bran	670.2 ± 13.3	nd	83.1 ± 1.6	13.2 ± 1.2	17.9 ± 1.2	nd	nd
	PP	661.1 ± 21.1	211.1 ± 15.7	12.1 ± 4.0	45.1 ± 4.4	87.9 ± 8.4	3.1 ± 0.4	0.1 ± 0.0
	PP + WB	675.1 ± 4.0	294.9 ± 3.7	82.1 ± 2.0	12.4 ± 5.2	17.9 ± 1.5	3.6 ± 0.4	0.4 ± 0.0

Note: The initial number: *T. molitor*, 300; *Z. atratus*, 250. nd = not determined.
[a] Cannibal rate was calculated based on the number of disappeared larvae divided by initial number of the larvae.
[b] Specific PP consumption rate was calculated on the basis of the mass of PP consumed over the test period (35 days) and the initial number of larvae.

2.4. Characterization of PP biodegradation with egested frass

Analyses of WCA of the PP foam and frass samples from PP-fed larvae were performed using Dataphysics DCAT21 (Heilna Trade GmbH, Germany). Thermal analysis of the PP foam and frass samples was conducted with TGA (TGA/SDTA851e, Mettler Toledo, U.S.A.). The samples were heated from 30 to 900 °C at a rate of 10 K/min under a high-purity nitrogen ambience (99.999%).

The frass samples were collected on day 35 for HT- GPC analysis (PL GPC 220, Agilent Technologies, Inc., USA) to determine M_n, M_w and M_z of the residual PP versus PP foam. Frass sample (100 mg) for each treatment group was collected, dried and gently crushed in a mortar and pestle. Extraction of residual PP from the samples was conducted by dissolving PP in 4 mL 1, 2, 4-trichlorobenzene (1, 2, 4-TCB) solvent (Honeywell International, Inc., USA) with the extraction of 4-h under heating condition. Then 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 re-dissolved 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. Polydispersity index (PDI) was calculated as PDI = M_w/M_n.

FTIR spectra of the PP foam and frass samples from PP-fed larvae were obtained using a Nicolet iS50 FTIR spectrometer (Thermo Fisher Scientific, U.S.A.). 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 sample was PP foam. Samples from frass (10 mg) of PP-fed larvae were gently crushed in a mortar 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 (version 10.0.2).

2.5. Microbial community analysis

At the end of the 35-d experiment, the gut tissues of the larvae (30 in each group) were randomly collected for microbial community analysis. The unfed larvae and the larvae fed with wheat bran were used as controls. The larval samples of antibiotic treatment were collected on day 14 for comparison of gut microbial communities between gentamicin treated versus control. According to the manufacturer's instruction, total bacterial genomic DNA samples were extracted using Fast DNA SPIN extraction kits (MP Biomedicals, Santa Ana, CA, USA). The DNA extraction and PCR amplification methods were performed as described previously (Lou et al., 2020). Phasing amplicon sequencing was applied to sequence the V3 − V4 region of the 16S rRNA gene, and then purified amplicons were paired-end sequenced on an Illumina MiSeq platform. The low-quality sequences were filtered through following criteria (Gill et al., 2006; Chen and Jiang, 2014). Operational Taxonomic Units (OTUs) were clustered using UPARSE (v9.2.64) with 0.97 identity threshold and chimeric sequences were 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%. Alpha diversity, beta diversity, and a differential abundance analysis were conducted based on the OTU sequences and taxonomic ranks using QIIME (v1.9.1) (Caporaso et al., 2010) and R packages (v3.4.3). Principal component analysis (PCA) was conducted based on the genus-level compositional profiles (Ramette, 2007).

2.6. Statistical analysis

Statistical analyses were performed in IBM SPSS Statistics (version 25). To evaluate the differences in PP consumption, survival, changes in molecular weights, and microbial diversity, ANOVAs were performed, followed by pairwise comparisons using Student's *t*-test with Tukey's correction to assess differences between diets and species. All *p*-values are adjusted *p* values and all error values are average ± standard deviation.

3. Results and discussion

3.1. PP consumption by T. molitor and Z. atratus larvae

The larvae of both *T. molitor* and *Z. atratus* chewed and penetrated into PP foams as they do with expanded polystyrene foam (Yang et al., 2018a, 2018b) as shown in Fig. 1a and b. Due to larval size, *Z. atratus* larvae made much larger sized holes and tunnels in PP foams than *T. molitor* larvae did. With 1.0 g PP foam initially added, the mass of consumed PP foams increased progressively throughout the 35-day experiment period (Fig. 1c). The larvae fed PP + WB consumed much more PP foam than those fed PP foam only; *Z. atratus* larvae consumed much more PP foam than *T. molitor* larvae. The total PP mass consumed by *T. molitor* fed PP only and fed PP + WB was 96.1 ± 5.3 and 161.6 ± 9.2 mg, respectively, indicating that the co-diet bran enhanced PP consumption by 68.2%. Respective PP mass consumed by *Z. atratus* larvae fed with PP only and fed with PP + WB was 211.1 ± 15.7 mg and 294.9 ± 3.7 mg over the 35-day period, indicating that feeding co-diet also enhanced PP consumption by 39.7%. WB is a normal feedstock for yellow mealworms and superworms and can provide all nutrients for mealworms to complete their life cycle. Instead, PP contains only hydrogen and carbon elements, and does not provide necessary nutrition (N, P, Na, K, trace elements, amino acids, etc.) for a long-term survival and development. Therefore, although digestion of PP can provide energy source for life activities and to maintain a SR higher of the larvae fed with PP only than that of unfed larvae in short term (3–5 weeks) but larval weight was decreased due to consumption of body biomass because of the lack of nitrogen sources and other nutrients. The addition of co-diet WB relieved this constraint. *T. molitor* and *Z. atratus* larvae obtained nutrition needed from WB to synthesize enzymes and digestive reagents, and thus PP consumption activities were enhanced, resulting in approximately doubled the consumption rates than that of PP fed only (Fig. 1c). Similar observations were observed previously during biodegradation of PS and LDPE by *T. molitor* and *Z. atratus* larvae (Yang et al., 2018a, 2018b; Brandon et al., 2018; Peng et al., 2020a; Yang et al., 2021).

The changes in survival rates (SRs) of the larvae of eight treatment groups over 35 day at 25 °C are presented in Fig. 1d. The SR of *T. molitor* larvae fed PP only was 88.7 ± 0.7%, significantly ($p < 0.01$) greater than that of unfed controls (68.4 ± 1.0%), and slightly ($p < 0.05$) lower than those of WB-fed larvae (90.1 ± 0.4%) and PP + WB (91.0 ± 0.3%). The SRs of *Z. atratus* in all groups were lower than those of *T. molitor* under the same feeding conditions, likely due to cannibalistic behavior of *Z. atratus*. SR of PP + WB group (82.1 ± 2.0%) was similar to that of *Z. atratus* fed with WB only (83.1 ± 1.6%), and both were much higher ($p < 0.01$) than those of the unfed control (37.2 ± 4.2%) and sole PP-fed group (12.1 ± 4.0%), which was the lowest among all treatment groups.

Z. atratus larvae tend to cannibalize the pupae and larvae of their own species (Tschinkel, 1981). Cannibalization was observed in both *T. molitor* and *Z. atratus* larvae during the tests. *Z. atratus* larvae had much higher cannibalism rate (CR) than *T. molitor* larvae in

corresponding groups (Fig. 1 and Table 1), e.g., the CRs of the larvae fed with WB were 13.2 ± 1.2% versus 1.7 ± 0.3% for *Z. atratus* versus *T. molitor*. When the larvae were fed with PP only, the larvae did not get enough energy from consumption of PP, resulting in higher cannibalism rates and lower SR, especially for *Z. atratus* larvae fed with PP only (12.1 ± 4.0%) than those fed with WB (83.1 ± 1.6%) and WB + PP (82.1 ± 2.0%) (Fig. 1 and Table 1). During 35-day test period, except the dead larvae remained in the incubators, the numbers of *Z. atratus* larvae in PP-fed only group and the unfed group decreased continuously compared with those fed with WB and PP plus WB, resulting in lower SRs and CRs due to lack of normal food (Table 1). When PP was applied as the sole diet, PP consumption rate and SRs of both *T. molitor* and *Z. atratus* larvae were lower than those fed with PP plus WB, indicating that the larvae feeding PP only obtained energy from digestion of PP as well as by consuming dead worms and their molts, which were not sufficient to support their growth and live development. The similar observations were reported in the studies on biodegradation of PS and LDPE foam by *T. molitor* and *Z. atratus* previously (Peng et al., 2020a).

In this study, the SRs of the *Z. atratus* larvae fed with PP foam only was similar to that of unfed larva on day 21 (73.1 ± 1.7% versus 78.3 ± 3.5%, Fig. S2), and then become significantly lower than that of unfed larvae on day 35 (12.1 ± 4.0% versus 37.2 ± 4.2%, Table 1). The observation on day 35 is different from those reported during biodegradation of PS and LDPE foams by *Z. atratus* using young larvae with around 130 mg each larva (Peng et al., 2020a, 2020b) i.e., SRs of the larvae fed with PS and LDPE were much higher than that of unfed larvae. The unusual observation could be attributed to cannibalism among *Z. atratus* larvae, especially mature or large-sized larvae. On the other hand, in *T. molitor* groups, SRs of the larvae fed with PP only were much higher than that of unfed larvae (88.7 ± 0.7% versus 68.4 ± 1.0%) as similar as observed previously during biodegradation of PS and LDPE by *T. molitor* (Yang et al., 2018a; Brandon et al., 2018; Yang et al., 2021). The lower SR of *Z. atratus* larvae fed PP only than that of unfed larvae was likely attributed to the higher death rate (87.9 ± 8.4% versus 62.8 ± 1.2%) and higher cannibal rate (45.1 ± 4.4% versus 59.3 ± 3.3%, Table 1) in this study. Large-sized mature *Z. atratus* larvae have strong cannibalism behavior under nutrition limiting condition. In this study, we used mature larvae (average weight of around 630 mg per larva), which showed cannibalism aggressively. It remained unknown if any PP biodegraded intermediates caused higher death rate of the *Z. atratus* larvae fed with PP only than unfed larvae and also stimulated cannibalism among *Z. atratus* larvae. Further research is needed to characterize the mechanism.

Averaged SPPCR are summarized in Table 1. On the basis of larval numbers, *Z. atratus* larvae exhibited much higher PP consumption rates than *T. molitor*, i.e., respective 3.1 ± 0.4 versus 1.0 ± 0.4 mg·100 larvae^{-1} d^{-1} for the larvae fed with PP only, ±and 3.6 ± 0.4 versus 1.6 ± 0.3 mg·100 larvae^{-1} d^{-1} for the larvae fed with PP + WB (Fig. 1e and f). The higher rates of *Z. atratus* than *T. molitor* larvae were likely attributed to the difference of larval size (approximately 60 mg versus 670 mg per larva). When the specific PP consumption rates were calculated on the basis of larval weight (SPPC-W, mg PP·g larva^{-1} d^{-1}), the SPPC-Ws of *Z. atratus* groups fed PP only versus WB + PP were 0.1 ± 0.0 and 0.4 ± 0.0 mg PP·g larva^{-1} d^{-1}, respectively; while *T. molitor* showed 0.2 ± 0.1 and 0.3 ± 0.0 mg PP·g larva^{-1} d^{-1}, respectively (Table 1). Because that the weight of *Z. atratus* was about 10 times *T. molitor*, the SPPC-Ws of *T. molitor* were higher than *Z. atratus* (Table 1 and Fig. 1f). The data indicated that co-diet bran enhanced PP consumption as observed in the tests of biodegradation of PS with *T. molitor* (Yang et al., 2018a; Brandon et al., 2018) and *Z. atratus* larvae (Peng et al., 2020a).

3.2. Evidence of PP depolymerization

Depolymerization is the first and essential step in the biodegradation of plastics. The depolymerization and biodegradation of ingested PP in larval gut were characterized using the egested frass from *T. molitor* and *Z. atratus* larvae that contained extractable fraction (residual PP polymer) which was composed of non-degraded polymer and partially degraded polymer, and non-extractable fraction which contained solid intermediates and other undigested residues (Brandon et al., 2018; Yang et al., 2018a). The PP foam was used as control.

GPC results indicated significant changes in M_n, M_w and M_z i.e., an increase in M_n, a decrease in M_w, and a significantly ($p < 0.01$) decrease in M_z after the ingested PP passed through the digestive system of both *T. molitor* and *Z. atratus*. For *T. molitor* and *Z. atratus* larvae, M_n values of the residual polymers increased from 109.8 kDa to 123.0 kDa by 12.1% and to 177.3 kDa by 61.5%, respectively; M_w decreased from 356.3 kDa to 283.6 kDa by 20.4% and to 324.1 kDa by 9.0%, respectively; M_z decreased from 765.0 kDa to 506.7 kDa by 33.77% and 520.4 KDa by 32.0%, respectively (Fig. 2a and Table S3). Analysis of the distribution of accumulated molecular number portion (Ht, %) versus molecular weight (Fig. 2b) indicated a clear shift of molecular weight distribution to a large molecular weight side. The residual PP in both *T. molitor* and *Z. atratus* frass samples tended to decrease polymer portion by 10–100 kDa.

According to previous studies (Brandon et al., 2018; Peng et al., 2019; Yang et al., 2015a, 2015b, 2018a, 2018b, 2020), two depolymerization patterns i.e., broad depolymerization (i.e., a decrease in both Mw and Mn simultaneously) and limited extent depolymerization (i.e., an increase in M_n or in both M_n and M_w) occur during biodegradation of plastic polymers. To date, the results of PS biodegradation by *Tenebrio* genus showed only broad depolymerization pattern (Brandon et al., 2018; Peng et al., 2019; Yang et al., 2015a, 2015b, 2018a, 2018b) but LDPE biodegradation by *T. molitor* showed both broad and limited extent depolymerization patterns (Yang et al., 2020). *Z. atratus* larvae performed both depolymerization patterns for PS biodegradation and limited extent depolymerization for LDPE (Peng et al., 2020a). In this study, the changes in Mn and Mw of PP after passage via the larval gut of both *T. molitor* and *Z. atratus* showed an increase in Mn from 109.8 kDa to 123.0 kDa and to 177.3 kDa, respectively, but decreases in Mw and Mz, indicating a limited depolymerization pattern during PP biodegradation by both *T. molitor* and *Z. atratus*. The observation of limited extent depolymerization has also been reported recently by other researchers, e.g., PUR biodegradation by a landfill microbial culture (Gaytán et al., 2019) resulted in an increase in Mw from 208.5 to 229.4 kDa and then decrease to 169.9 kDa; PS degradation in *Galleria mellonella larvae* (Lou et al., 2020) with an increase in Mn from 132.1 kDa to 146.9 kDa as well as in Mw from 361.7 to 377.8 kDa; and an increase in both M_n and M_w of residual PS after digestion of PS foam by land snails *Achatina fulica* (Song et al., 2020). In this study, the limited extent depolymerization was likely related to the selective depolymerization and degradation of lower molecular polymers at higher rates than large molecular portion because M_z was reduced. The results also indicated the complexity and limitation of biodegradation of PP in plastics-eating *T. molitor* larvae and *Z. atratus*.

Polydispersity index (PDI) is a measure of the distribution of molecular mass in a given polymer sample, as well as polymer diversified indication. PDI of the extracted polymers from frass of *T. molitor* (2.3) and *Z. atratus* (1.8) was significantly ($p < 0.01$) lower than the PP control (3.2). The decrease in PDI indicated less broadness in the molecular weight of the residual polymer. In this study, the decrease in PDI appeared due to the increase of M_n i.e., reduction of lower molecular weight portion (Table S3).

3.3. Evidence of polypropylene oxidation/biodegradation

Characterization of oxidation and biodegradation of PP was performed with WCA, TGA, FTIR, and ^{1}H MNR, which are established methods widely used to investigate plastic biodegradation including PP (Pires et al., 2019), PS and PE (Brandon et al., 2018; Yang et al., 2018a, 2018b; Peng et al., 2019, 2020a; Yang et al., 2021) as well as rubber (Aboelkheir et al., 2019).

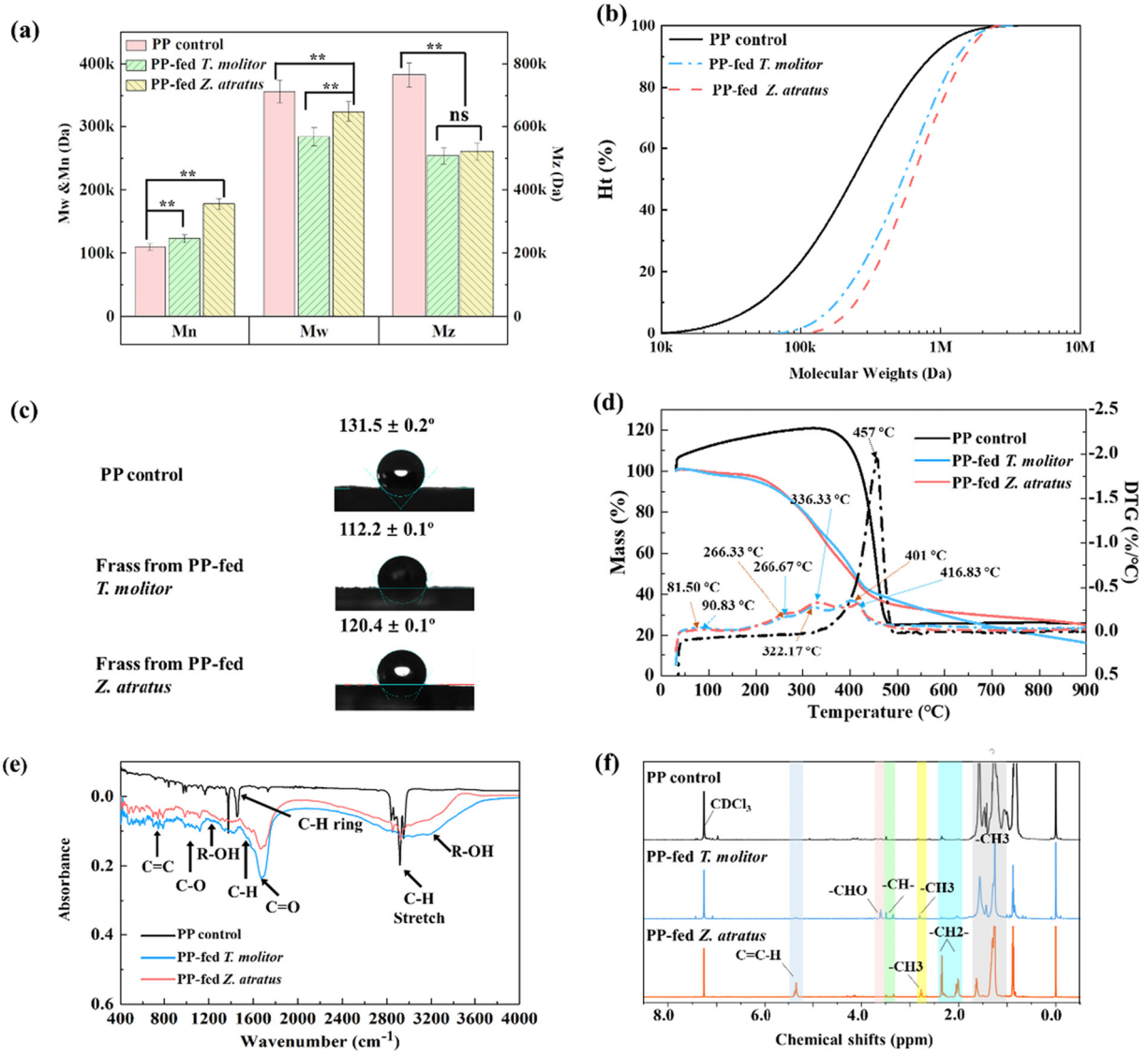

Fig. 2. Characterization of PP depolymerization/biodegradation in *T. molitor* and *Z. atratus* larvae. (a) Comparison of Mw, Mn, and Mz of PP feedstocks and the frass of *T. molitor* and *Z. atratus* fed with PP only. (All values represent mean ± SD, n = 3. Significance (Student's t-tests) $p < 0.05$ indicated by *, $p < 0.01$ indicated by **, and no statistical significance indicated by ns.) (b) Changes in molecular weight distribution of PP feedstock and frass of *T. molitor* and *Z. atratus* fed with PP only. Samples were obtained on day 35. (c) WCA of the control PP feedstock and the frass of *T. molitor* and *Z. atratus* fed with PP only. (d) TGA spectra of the control PP feedstock and the frass of *T. molitor* and *Z. atratus* fed with PP only. (e) FTIR spectra of the control PP feedstock and the frass of *T. molitor* and *Z. atratus* fed with PP only. (f) ^{1}H NMR spectra of the control PP feedstock and the frass of *T. molitor* and *Z. atratus* fed with PP only.

Changes in surface hydrophobicity of the egested frass from *T. molitor* and *Z. atratus* fed with PP as sole diet are the result of biodegradation occurring on the polymer surface. The hydrophobic polymer surface prohibits effective adsorption and catalytic performance of polymer-degrading enzymes (Wei and Zimmermann, 2017). Decrease in hydrophobicity helps enzymatic attacks on the C—C bonds and vice versa. The WCA of control PP foam was 131.5 ± 0.2° ($n = 3$). The WCAs of frass from PP-fed *T. molitor* and *Z. atratus* larvae were 112.2 ± 0.1° and 120.4 ± 0.1° (n = 3), respectively, which was significantly ($p < 0.01$ and $p < 0.05$) lower than those of the control sample (Fig. 2c). The decreased WCAs compared with the control indicated the reduction of hydrophobicity of polymer surface, which is a sign of surface modification and possible biodegradation as observed in microbial biodegradation of LDPE (Sudhakar et al., 2008; Yang et al., 2014). The WCA of frass from *T. molitor* larvae was lower than that of *Z. atratus* larvae (Fig. 2c), suggesting that *T. molitor* performed PP surface modification superior to *Z. atratus*.

Thermal modifications of frass samples from PP-fed *T. molitor* larvae and *Z. atratus* larvae were detected using TGA after the 35-day test. As shown in Fig. 2d, the TGA indicated an obvious mass loss in the virgin PP foam that occurred from 370 to 480 °C, with about 92.55% of weight loss occurring during a single stage, and the maximum decomposition rate occurred at 457 °C. By comparison, four maximum decomposition rates appeared at 90.83, 266.67, 336.33, and 416.83 °C for frass from *T. molitor* fed PP only. Accordingly, four maximum decomposition rates appeared at 81.50, 266.33, 322.17, and 401.00 °C for frass from PP-fed *Z. atratus*. The decomposed part under 100 °C was classified as volatile organics, including gut secretion, and some degradation products from PP biodegradation e.g., carboxylic acid compounds, as similarly observed and reported in previous studies on the biodegradation of PS in *T. obscurus* larvae (Peng et al., 2019) and *Z. atratus* larvae (Peng et al., 2020a). The decomposed parts in the ranges of 100–370 °C might be attributed to other biological digestive products from cannibalism and PP biodegradation residues from the guts of both species. Additionally, the mass loss ratios of the frass from *T. molitor* and *Z. atratus* larvae in the stage of 370 to 480 °C were respectively 23.23% and 24.49%, much lower than that of the PP foam feedstock (92.55%), indicating that the frass contained not only residual PP

polymer but also more new biodegraded products. This result confirmed the modification and degradation of PP polymer as it passed through the guts of *T. molitor* and *Z. atratus* larvae. The different maximum decomposition rates observed in TGA analyses further suggested that different degradation pathways of PP polymers might occur in the larval guts of the two insect species.

To determine the oxidation and biodegradation of ingested PP polymer, at the end of the 35-day test, the frass samples from the larvae fed with PP only were analyzed with FTIR. A comparison of FTIR spectra of the PP foam and frass samples indicated chemical changes and the formation of new functional groups in the frass samples due to biodegradation (Fig. 2e). The spectrum of frass from *T. molitor* fed with PP only was similar to that from *Z. atratus*, but with higher intensities in the peaks associated with new functional groups. In the control PP foam feedstock, absorption peaks at 2839–2951 cm^{-1} were attributed to the C—H alkyl stretch of PP polymer (Auta et al., 2018), whereas the intensities of these peaks were much weaker in the spectra of the frass samples of both *T. molitor* and *Z. atratus*. Likewise, the peaks at around 809 cm^{-1}, 841 cm^{-1}, 899 cm^{-1}, and 1500 cm^{-1}, assigned to the C—H alkyl bend and the aromatic structures C—H ring had high intensities in the PP foam (Auta et al., 2018), but they disappeared in all the frass samples. The appearance of new peaks at around 1710–1750 cm^{-1} and 1167 cm^{-1} were attributed to the -C=O carbonyl and -C–O stretch as described previously (Yang et al., 2014; Bombelli et al., 2017; Auta et al., 2018; Peng et al., 2020a), were formed in the frass samples of both insect species, confirming the incorporation of oxygen into the PP polymer chains due to oxidation. Additionally, a new absorption peak appeared in the frass samples at around 3310 cm^{-1} attributable to the O—H of the hydroxyl band, suggesting that the surface had a change from a hydrophobic to a more hydrophilic property (Yang et al., 2018a; Peng et al., 2019). The FTIR data with higher intensity in hydroxyl groups in the frass spectra of *T. molitor* was consistent with the results from WCA (Fig. 2c and e). The formation of carbonyl (C=O) and C—O groups observed with FTIR were reported during iPP degradation in soil with enzymatic additive (Pires et al., 2019). FTIR results of this study were also similar to previously reported data on the biodegradation of PS and PE polymers in *T. molitor*, *T. obscurus* and *Z. atratus* larvae (Brandon et al., 2018; Yang et al., 2018a, 2019b; Peng et al., 2019, 2020a).

Comparison of ^{1}H NMR spectra of the PP foam with the spectra of residual PP extracted with D-Chloroform from the frass revealed the formation of new peaks in the frass extracts from *T. molitor* and *Z. atratus* larvae fed PP only (Fig. 2f). Additional peaks and obvious chemical shifts in polymer residues of frass from PP-fed *T. molitor* and *Z. atratus* were observed in the range of 1.0–2.0 ppm, denoting the formation of CH_3 moiety due to the breakdown of long chain polymers (Skariyachan et al., 2018) and/or release of methyl groups from subunit of the polymer backbone after biodegradation. Similarly, a new peak at around 2.78 ppm assigned for metabolites e.g. amino acids (Zainal et al., 2019) was identified in the ^{1}H NMR spectra of frass extracts from both frass samples. Moreover, new peaks in polymers from frass samples observed in regions between 2.9 and 3.9 ppm were attributed to the CH_2 groups next to the carbonyl group of the linker (Peng et al., 2008), revealing new peaks associated with the incorporation of oxygen, as can be observed in FTIR spectra (Fig. 2e). Different from the ^{1}H NMR spectra of *Z. atratus* group, the spectra of the frass from *T. molitor* larvae showed the presence of additional peaks around 3.5–4.0 ppm (-CHO, aldehyde moiety) which was absent in the control. For the PP-fed *Z. atratus* larvae, a new sharp peak appearing at around 2.34 ppm in the frass sample was evidence of chemical modifications resulting from biodegradation process of polyolefin plastic (Kundungal et al., 2019). Likewise, a new peak around $\delta_H = 5.3$ ppm in a region associated with alkene bonds (C=C − H) was found in frass extracts from larvae of *Z. atratus*, confirming the biodegradation of PP polymer (Brandon et al., 2018). The results of ^{1}H NMR analysis supported the findings of the formation of biodegraded intermediates from FTIR results (Fig. 2e). Differences in chemical shifts observed in this study suggested that the biodegradation pathways of PP polymers by *T. molitor* larvae might be different from that of *Z. atratus* larvae.

3.4. Effect of gut microbe suppression on PP depolymerization/biodegradation

To examine the effect of gut microbiota on PP degradation, antibiotic suppression tests were conducted to investigate the response of PP depolymerization and biodegradation in *T. molitor* and *Z. atratus* larvae. Gentamicin sulfate is active against a wide range of bacterial infections, mostly Gram-negative bacteria and certain Gram-positive bacteria, and therefore was selected for the gut microbiota depression (Yang et al., 2015b; Yang et al., 2018a; Peng et al., 2020a; Yang et al., 2021). The results showed a strong ability to decrease the number of gut microbes after 7 days from 3.2×10^6 to 9.2×10^4 for *T. molitor* and from 8.9×10^6 to 1.3×10^5 for *Z. atratus*, and maintained suppression of the gut bacteria afterwards. After 14 days of gentamicin suppression, bacteria numbers decreased by two to three magnitudes compared to controls (Fig. S1a and b), implying that the gut bacteria were effectively suppressed. Subsequently, the frass samples from antibiotic gentamicin suppression groups were collected for HT-GPC analysis. The results indicated that both *T. molitor* and *Z. atratus* larvae lost their ability of depolymerization of PP, thus biodegradation capacity, when gut microbes were severely inhibited (Fig. S1c and d). M_w and M_n were unchanged i.e., no statistically significant differences between PP foam and residual PP polymers extracted from the frass of gentamicin suppression groups, indicating that the PP depolymerization/biodegradation by both *T. molitor* and *Z. atratus* is gut-microbe dependent. Previous studies indicated the gut-microbe dependence of PS depolymerization in *T. molitor* (Yang et al., 2015b; Yang et al., 2018a; Yang et al., 2021) and *Z. atratus* (Peng et al., 2020a) as well as *T. obscurus* (Peng et al., 2019), but LDPE depolymerization was less-dependent or independent in *T. molitor* larvae (Yang et al., 2021). Previous studies also indicated that *T. molitor* larvae are capable of removal or digestion of lignin materials in rice straw, corn straw and wheat straw (Yang et al., 2019a, 2019b). Although the capacity of lignin-degradation by *Z. atratus* is unknown we hypothesize that the degradation of PP as well as PS and LDPE by *T. molitor* and *Z. atratus* may be related to their lignin-degrading capacity.

Further investigation is needed to verify the ubiquity of microbe-dependence for PP depolymerization with the larvae from different sources and lower molecular weight PP materials. The mechanism of the microbe-dependence should also be further investigated.

3.5. Gut microbial analysis

Analyses of microbial diversity in *T. molitor* and *Z. atratus* larvae fed with three different diets i.e., WB fed only, PP fed only, and PP + WB co-fed were conducted (Table 2). The DNA extraction and PCR amplification methods were similar to that reported previously (Brandon et al., 2018) and are detailed in Supporting Information (SI, M1). A total of 584,624 sequences were obtained from all six groups with coverage of 99% (Table 2). The number of OTUs of the groups of *T. molitor* and *Z. atratus* of PP-fed (101 and 99) and PP + WB co-fed (112 and 122) groups were lower with those of WB-fed (152 and 151), indicating that feeding PP significantly reduced gut community diversity. Shannon and Simpson indices also indicated that species richness slightly decreased after *T. molitor* larvae received PP diets but *Z. atratus* larvae did not (Table 2).

A PCA based on OTU and heatmap based on Bray-Curtis distances revealed clusters associated with the different diets (Fig. 3a and b). The microbiomes of *T. molitor* larvae fed with PP and PP + WB were similar, and in a different cluster than the WB-fed group. A similar clustering was also observed in *Z. atratus* groups, indicating that the composition of microbial communities was distinct from larvae feeding on normal

Table 2
Bacterial diversity analyses based on illumine sequencing of the 16S rRNA Gene amplicons for *T. molitor* and *Z. atratus* larvae under different diet conditions (WB only, PP only, and PP + WB groups).

Samples	OTU	Tag number	Chao1	observed	Shannon	Simpson
WB fed *T. molitor*	152	103,786	199.9	127.2	2.7	0.8
PP fed *T. molitor*	101	116,108	157.5	81.2	1.5	0.5
PP + WB co-fed *T. molitor*	112	85,429	178.7	105.5	1.2	0.3
WB fed *Z. atratus*	151	72,408	198.0	151.0	3.3	0.9
PP fed *Z. atratus*	99	103,279	123.6	88.8	3.0	0.8
PP + WB co-fed *Z. atratus*	122	103,614	156.2	107.5	3.2	0.8

diet bran and PP containing diets, and also distinct between species i.e., *T. molitor* and *Z. atratus*.

Relative abundance analysis revealed that the predominant phylum in the guts of *T. molitor* larvae in all diets was *Proteobacteria* (Fig. 3c). But a significant difference in predominant phyla was observed between the gut microbiomes of *Z. atratus* larvae fed WB and PP. The predominant phyla belonging to *Proteobacteria* was found in *Z. atratus* larvae fed PP only and PP + WB, and *Firmicutes* was in larvae fed WB only. For gut microbiomes of *T. molitor* and *Z. atratus* larvae, the phylum *Proteobacteria*, which was also the predominant phyla associated with feeding LDPE in greater waxworms, larvae of *Galleria mellonella* (Lou et al., 2020).

Further analysis at genus level indicated that the core microbiomes were markedly different between the *T. molitor* and *Z. atratus* larvae after feeding PP (Fig. 4a). The predominant populations in the guts of PP-fed and PP + WB co-fed *Z. atratus* larvae were affiliated with *Citrobacter* sp. (relative abundance of 31.32% and 31.06%, respectively) and *Enterobacter* sp. (relative abundance of 25.30% and 20.36%, respectively). Both *Citrobacter* sp. and *Enterobacter* sp. were observed during LDPE-degradation (Yang et al., 2014; Brandon et al., 2018), and they shifted to higher relative abundances in PP-fed diet, suggesting that these genera were significantly related to PP degradation in *Z. atratus* larvae. *Citrobacter* sp. which is strongly associated with LDPE degradation by utilizing oxygen (Brandon et al., 2018), could be involved in PP degradation through the incorporation of oxygen into the PP polymer chains. *Kluyvera* sp. was also a dominant genus in the gut microbiome of *T. molitor* larvae fed PP and PP + WB, with respective 70.57% and 81.99% relative abundances, which increased by 56.14% and 67.56% compared with the larvae fed WB only (relative abundance 14.43%) (Fig. 4a). The genus *Kluyvera* sp. was found colonizing cinematographic films (plastic materials), resulting in the cinematographic film biodeterioration and chemical/mechanical degradation (Abrusci et al., 2005). In the guts of PP-fed and PP + WB co-fed *T. molitor* larvae, *Kluyvera* sp. showed overwhelming superiority compared with that of the WB-fed group, suggesting that this genus might play a significant role in PP degradation in the *T. molitor* larval gut. Notably, all three predominant populations are members of *Enterobacteriaceae*, a family known to contain PE-degrading bacteria (Yang et al., 2014). After PP feeding, community shift was mainly associated with the distribution of *Enterobacteriaceae* (Fig. 3d); this phenomenon was consistent with the results of studies on PS or PE biodegradation (Peng et al., 2019). The role of *Citrobacter* sp., *Enterobacter* sp., and *Kluyvera* sp. should be further verified by isolation of single cultures and enzymatic tests to determine plastic depolymerizing capacity and the enzyme(s) involved.

To further assess whether particular OTUs were associated with different diets, comparison of differential abundance analyses was applied between different groups: PP-fed vs. WB-fed and PP-fed vs. PP + WB-fed (Fig. 4b–e). In addition to *Kluyvera* sp., differential abundance analysis revealed that *Lactococcus* sp., *Citrobacter* sp., *Enterobacter* sp., and *Spiroplasma* sp. exhibited obvious higher relative abundance in the PP-fed group when WB-fed and PP + WB co-fed microbiome from *T. molitor*

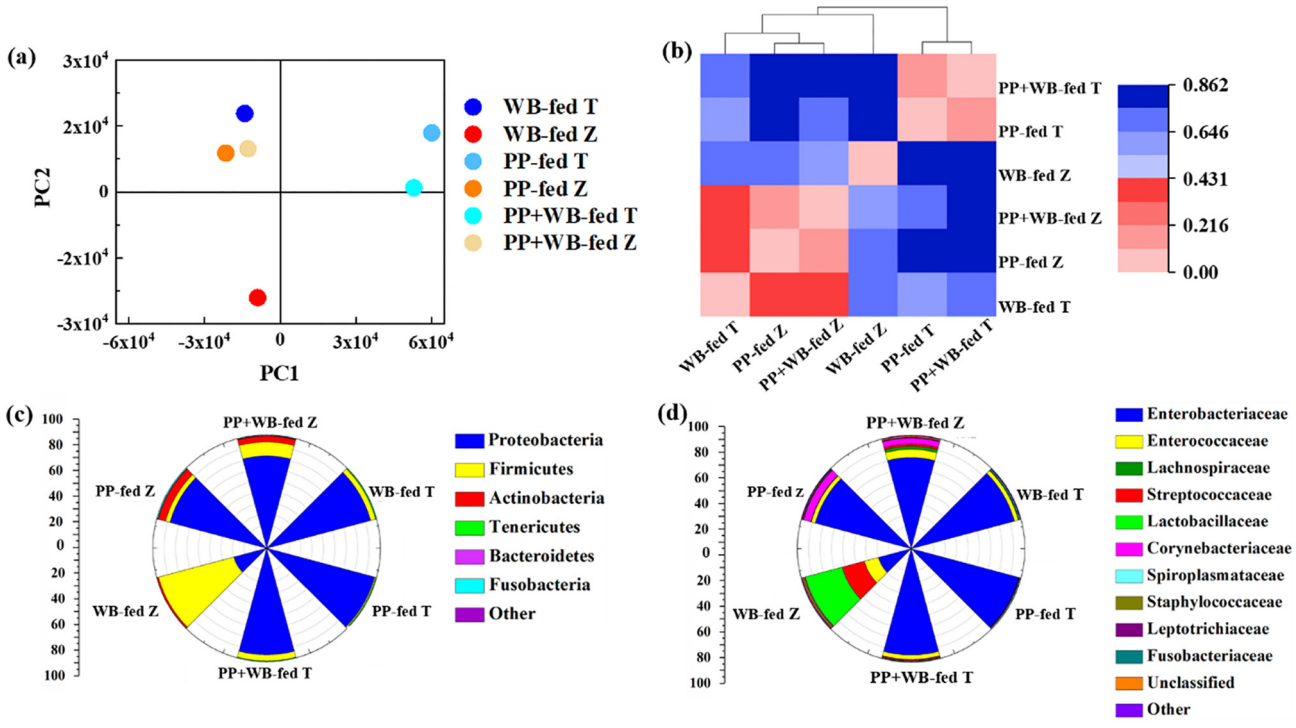

Fig. 3. Shift of gut microbial structure due to different diet conditions. (a) Gut microbial community structure analysis of the three different diets (WB fed only, PP fed only, and PP + WB co-fed) by *T. molitor* and *Z. atratus* larvae. Principal component analysis and (b) community diversity distance analysis based on Bray-Curtis distances. Relative abundance of dominant populations in the gut microbiomes of *T. molitor* and *Z. atratus* larvae fed the different diets at (c) the phylum and (d) the family levels. WB-fed T = *T. molitor* larvae fed with WB only; PP-fed T = *T. molitor* larvae fed with PP only; PP + WB-fed T = *T. molitor* larvae co-fed with PP + WB; WB-fed Z = *Z. atratus* larvae fed with WB only; PP-fed Z = *Z. atratus* larvae fed with PP only; PP + WB-fed Z = *Z. atratus* larvae co-fed with PP + WB; PP = polypropylene; WB = wheat bran.

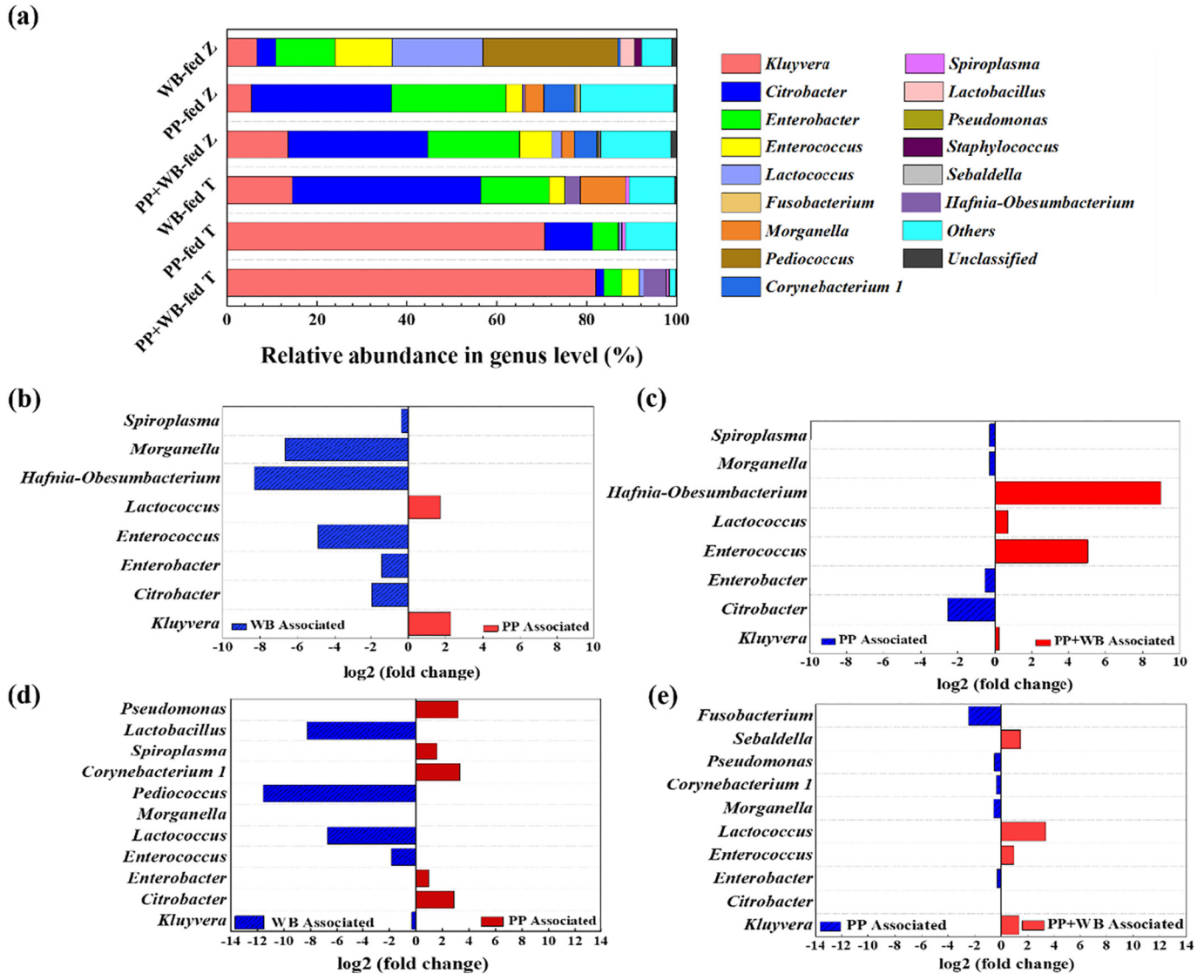

Fig. 4. Analysis of gut microbiomes of *T. molitor* and *Z. atratus* larvae. (a) Relative abundance of dominant populations in the gut microbiomes of *T. molitor* and *Z. atratus* larvae under different diet conditions (WB only, PP only, PP + WB, and unfed) at the genus levels. Differential relative abundance of the predominant populations in the guts of *T. molitor* versus *Z. atratus* larvae fed with different diets: (b) WB-fed versus PP-fed gut microbiome of *T. molitor* larvae. (c) PP-fed versus PP + WB-fed gut microbiome of *T. molitor* larvae. (d) WB-fed versus PP-fed gut microbiome of *Z. atratus* larvae. (e) PP-fed versus PP + WB-fed gut microbiome of *Z. atratus* larvae. Samples were obtained on day 35 at the end of the test.

groups were used as the comparisons (Fig. 4b and c), indicating that these genera were significantly related to PP-fed diet. Similarly, *Citrobacter* sp., *Enterobacter* sp., and *Spiroplasma* sp., with proven capacity for plastic degradation (Yang et al., 2014; Brandon et al., 2018), were also found to be associated with a PP diet in the gut microbiome of *Z. atratus* larvae (Fig. 4d and e). *Corynebacterium* sp. and *Pseudomonas* sp. with proven association to the biodegradation of synthetic polymeric materials, e.g. PE or PS (Gu, 2007; Brandon et al., 2018; Lou et al., 2020), also exhibited significant differences associated with the PP diet in *Z. atratus* groups.

The gut community analysis of the larvae fed with PP in the presence and absence of gentamicin depression versus those fed with WB indicated significant differences among those three diet conditions (Fig. S4a). In general, all predominant bacterial species were decreased significantly even PP was fed in the presence of gentamicin. Suppression of gut microbes with antibiotic gentamicin resulted in not only decrease in gut microbial levels by 2–3 magnitudes but also decrease in gut bacterial species, e.g., decrease of *Enterococcus, Morgunella, Lactococcus, Spirolasma* and *Hufnia-Obesumbacterium* in *T. molitor*; and decrease of *Enterococcus, Morgunella, Lactococcus, Pediococcus*, and *Hufnia-Obesumbacterium* in *Z. atratus* (Fig. S4), indicating almost complete suppression of major gut microbes.

This study with Illumina sequencing of the 16S rRNA gene demonstrated that the gut microbiomes were shaped by different diets, resulting in a significant difference in community structures in both species.

4. Conclusions and outlook

This study is the first report on the consumption and biodegradation of PP by two plastic-eating insects, *Tenebrio molitor* and *Zophobas atratus* larvae. The results indicated that the ingested PP polymer with high average molecular weights (M_n, 109.8; M_w, 356.2; and M_z, 765.0 kDa) was depolymerized and biodegraded in their guts. The PP depolymerization and biodegradation is gut-microbe dependent. Further studies are needed to examine the ubiquity of the PP biodegradation and the impact of physical and chemical properties of PP materials (molecular weight, crystallinity, additives, etc.) on their biodegradability.

Z. atratus larvae showed higher PP consumption by number-averaged specific rate and lower rate by larval weight averaged specific rate than *T. molitor* larvae due to their size difference. Based on survival

rates (SRs), cannibalism rate (CRs), and extent of depolymerization and biodegradation, *T. molitor* larvae appeared superior to *Z. atratus* larvae in PP biodegradation. The data indicated that supplementation of co-diet bran enhanced the PP consumption rate similarly to previous observations on the biodegradation of PS and LDPE in *T. molitor*, *T. obscurus* and *Z. atratus*. The differences among these species could be associated to generic properties of the different larvae and their diversified gut microbial communities. Further studies should address the mechanisms of PP degradation in relation to the enzyme(s), larval and microbial genes, and natural diet behaviors of *T. molitor* and *Z. atratus*.

The results of GPC analyses indicated that PP biodegradation in both *T. molitor* and *Z. atratus* was performed via limited depolymerization pattern i.e., the increase in M_n, and decrease in M_w and M_z of the residual PP polymer in frass. This suggested that both larvae had capacity of preferred degradation of the portion of PP polymer with lower molecular weights.

The results of WCA, TGA, FTIR, and ^{1}H NMR confirmed oxidation and biodegradation of PP in both insect larvae, and also revealed that possible different degradation pathways of PP occurred in guts of *T. molitor* and *Z. atratus* larvae.

Antibiotic suppression tests indicated the gut microbe-dependence of PP depolymerization, which is similar to PS depolymerization but different from LDPE depolymerization in *T. molitor*. Further studies are needed to understand the mechanisms including responsible gut microbes, and functional enzyme(s) involved in depolymerization and the contribution of larval digestive system.

Application of next-generation sequencing to analyze of the gut microbiome revealed that two OTUs (*Citrobacter* sp. and *Enterobacter* sp.) were strongly associated with the PP-fed gut microbiome of *Z. atratus* larvae, while *Kluyvera* sp. was the predominant gut microbiome of *T. molitor* larvae fed PP. More studies will be needed to investigate these core microbes and their role in PP depolymerization and biodegradation.

Finally, the limited extent depolymerization and lower consumption rates by *T. molitor* and *Z. atratus* indicated the complexity and challenges of investigating PP biodegradation, even in darkling beetle larvae. *T. molitor* and *Z. atratus* larvae can serve as incubators or bioreactors to develop biotechnologies for the remediation and recycle of waste plastics like PP.

CRediT authorship contribution statement

Shan-Shan Yang: Conceptualization, Methodology, Supervision, Validation, Writing – original draft, review & editing, Funding acquisition. Meng-Qi Ding: Methodology, Formal analysis, Investigation, Methodology, writing - review & editing. Lei He: Methodology, Formal analysis, writing - review & editing. Chun-Hong Zhang: Investigation- sample collection & extraction. Qing-Xiang Li: Investigation-sample collection & extraction. De-Feng Xing: Conceptualization- experimental design. Guang-Li Cao: Conceptualization- experimental design. LeiZhao: Conceptualization- experimental design. Jie Ding: Conceptualization-experimental design. Nan-Qi Ren: Conceptualization- experimental design. Wei-Min Wu: Conceptualization, Methodology, Supervision, Validation, Writing – drafting, review & editing. All authors contributed to manuscript reviewing & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2020.144087.

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