

## Response of the yellow mealworm (*Tenebrio molitor*) gut microbiome to diet shifts during polystyrene and polyethylene biodegradation

Yu Lou <sup>a,1</sup>, Yiran Li <sup>a,b,1</sup>, Baiyun Lu <sup>a</sup>, Qiang Liu <sup>a</sup>, Shan-Shan Yang <sup>a</sup>, Bingfeng Liu <sup>a</sup>, Nanqi Ren <sup>a</sup>, Wei-Min Wu <sup>b</sup>, Defeng Xing <sup>a,\*</sup>

<sup>a</sup> State Key Laboratory of Urban Water Resource and Environment, School of Environment, Harbin Institute of Technology, Harbin 150090, China

<sup>b</sup> Department of Civil and Environmental Engineering, William & Cloy Codiga Resource Recovery Center, Center for Sustainable Development & Global Competitiveness, Stanford University, Stanford, CA 94305-4020, USA



### ARTICLE INFO

Editor: Dr. R. Teresa

**Keywords:**

Plastic biodegradation  
Mealworms  
Gut microbiome  
Plastic-degrading bacteria  
Diet shifting

### ABSTRACT

Plastic biodegradation by mealworm is regarded as an emerging strategy for plastic disposal. In this study, the polystyrene (PS) and low density polyethylene (LDPE) degradation efficiency by yellow mealworms (*Tenebrio molitor* larvae) supplemented with bran and the effects of plastics on the gut core microbiome were explored to construct a circular and continuous reactor for plastic biodegradation in the future. The gut microbiome was also investigated with dietary shift to explore the relationship between specific diets and gut microbes. The bran plus plastic (7:1 ratio, w/w) co-diet contributed to the mealworm survival and growth. The formation of  $-C^=O-$  /  $-C-O-$  groups in the plastic-fed mealworms frass represented the oxidation process of plastic biodegradation in the mealworm gut. The changes in molecular weights ( $M_w$ ,  $M_n$  and  $M_z$ ) of residual PS and LDPE in mealworms frass compared with that of PS and PE feedstock confirmed the plastic depolymerization and biodegradation. *Lactobacillus* and *Mucispirillum* were significantly associated with PE + bran diet compared to bran diet and PE diet, representing the response of mealworm gut microbiome to the bran and plastic mixture was distinguished from either bran or plastics alone. The gut microbiome changed substantially with the diet shift, indicating that microbial community assembly was a stochastic process and diverse plastic-degrading bacteria might occur in the mealworm gut.

### 1. Introduction

Plastic waste has become a worldwide concern because of the huge demand, ubiquity, and adverse environmental effects. The global annual rate of plastic production increases continuously to 368 million tons in 2019 (PlasticsEurope, 2020). However, most of these plastic products are designed for one-time use and are carelessly disposed of after their service period. Thus, plastic waste has spread from the land to the open ocean to the shorelines of remote islands and the deep sea (Barnes et al., 2009). The estimated global release of plastic waste is 9.5 million tons per year into the ocean, and the weight of plastic will eventually exceed that of fish by 2020 (Boucher and Friot, 2017; Wang et al., 2019). Furthermore, this accumulated plastic waste not only affects environmental aesthetic quality, but also poses a substantial threat to the health and survival of wildlife and humans (Compa et al., 2019). Plastic debris swallowed by wildlife affects normal eating behavior and disturbs

physiological functions (Derraik, 2002; Gregory, 2009). In addition, because of the existence of toxic raw material in plastics and the strong ability of plastics to absorb chemical pollutants from their surrounding environment, hazardous chemicals are transferred easily through the food web to all kinds of wildlife and humans through plastic waste (Rochman et al., 2013; Talsness et al., 2009).

Among the kinds of plastic wastes, polystyrene (PS) and polyethylene (PE) are of particular concern. PS and PE account for a large proportion of plastic waste and are widely used in our daily lives. PS is used for glass frames, plastic cups, and building insulation, comprised 7% of total plastic consumption, while PE, which includes linear low-density PE (LLDPE), low-density PE (LDPE) and high-density PE (HDPE), occupied 32% in 2015 and 39.8% in 2019 globally (PlasticsEurope, 2020). PS and PE are considered to be extremely persistent and their biodegradation in natural environments is in very low rate (Alexander, 1975). Therefore, photolysis or thermolysis pretreatments

\* Corresponding author.

E-mail address: [dxing@hit.edu.cn](mailto:dxing@hit.edu.cn) (D. Xing).

<sup>1</sup> These authors have contributed equally to this work.

are considered to be required before the plastics can be efficient biodegraded (Geyer et al., 2017; Guillet et al., 1974; Krueger et al., 2015). Over the past 50 years, a number of bacterial strains have been isolated for plastics biodegradation (Rucha V. Moharir, 2019; Sangale et al., 2012). However, some specific strains barely colonize untreated PE (Albertsson et al., 1987; Motta et al., 2009), and the bacteria capable of colonizing on plastic remain in a relative low rate.

The observation of mandibulate insects chewing and ingesting plastic bag promotes the investigation of plastic biodegradation via insects, and further provides the possibility that insect gut microbes might be a competitive source for screening plastic-degrading bacteria (Gotoh et al., 2015; Ng'ang'a et al., 2016). Particular insects or their gut microbes have been tested and verified to be capable of degrading polystyrene and polyethylene, including yellow mealworms (*Tenebrio molitor* larvae) (Yang et al., 2015a), dark mealworms (*Tenebrio obscurus* larvae) (Peng et al., 2019), superworms (*Zophobas atratus* larva) (Peng et al., 2020b; Yang et al., 2020), greater waxworms (*Galleria mellonella* larva), lesser waxworms (*Achroia grisella* larva) and Indian mealmoths worms (*Plodia interpunctella* larva) (Bombelli et al., 2017; Kundungal et al., 2019; Lou et al., 2020; Yang et al., 2014). The earthworm (*Lumbricus terrestris*), which belongs to the phylum of annelid, performs the PE particle size degrading (Lwanga et al., 2018) while land snails depolymerize and biodegrade PS foam (Song et al., 2020). Furthermore, polypropylene (PP), polyvinyl chloride (PVC), and polylactic acid (PLA) polymers have been confirmed to be degraded by the yellow mealworm (Peng et al., 2020a, 2021; Yang et al., 2021). The gut of yellow mealworm can also secrete emulsifying factors which assists the gut microbes and contributed to the plastic biodegradation (Brandon et al., 2021). In addition, more effective plastic-degrading bacteria and fungus have been isolated from the gut of larvae. *Enterobacter* sp. and fungus *Aspergillus flavus* have been isolated from the gut of the greater wax moth, which can be involved into PE degradation (Ren et al., 2019; Zhang et al., 2020). *Exiguobacterium* sp. strain YT2 isolated from yellow mealworm, *Pseudomonas* sp. isolated from superworm and *Acinetobacter* isolated from red flour beetle participate into PS degradation (Kim et al., 2020; Wang et al., 2020; Yang et al., 2015b).

Yellow mealworms from 12 sources in China, Northern Ireland, and the US have been verified capable of digesting PS foam, illustrating the ubiquity of PS degradation by mealworms (Yang et al., 2018a). Further, considering that a plastics-only diet lacks sufficient nutrients for protein synthesis to support larval growth, supplementary nutrients have been provided to help. In previous research, bran was supplemented but did not facilitate survival of mealworms. However, it did promote plastic consumption by larvae fed co-diets compared to those of mealworms offered plastic-only diets (Brandon et al., 2018). Research on the proportion of bran added has illustrated that PS consumption generally increases with a higher bran proportion (Yang et al., 2018b). The addition of extra nutrients might also be beneficial for the sustainable application of plastic biodegradation by mealworms. Therefore, more researches on the addition of extra nutrients to the plastic-fed insect are worthy of further investigating. Furthermore, the gut microbiomes of the mealworm and the greater wax moth larvae were investigated based on plastic diets (Brandon et al., 2018; Lou et al., 2020). Based on 16S rRNA gene sequencing, *Citrobacter* sp. and *Kosakonia* sp. were significantly related to both PS and PE diets of mealworms (Brandon et al., 2018), and *Bacillus* and *Serratia* were strongly associated with the PS and PE diets of the greater wax moth larvae, respectively (Lou et al., 2020). Though the gut microbial community of mealworms fed only PS or PE was investigated in several researches (Peng et al., 2019; Przemieniecki et al., 2020), the gut microbiome of mealworm fed PS/PE + bran has rarely been reported (Brandon et al., 2018). The gut microbial population is associated with the diet, so a dietary shift would affect the gut microbial community structure. Accordingly, we hypothesized that the gut microbiome of plastic-fed larvae might be dissimilarly shaped if a pre-diet (e.g., bran diet) were provided before the plastic diet. Exploring the mealworm gut microbiome under plastic-associated conditions

could help to understand the depolymerization mechanism of plastics by mealworm and its gut microbes.

Therefore, the shifts in the gut core microbiome with the dietary shift were monitored to evaluate the response of the gut microbial community to changes in diet in this study, in which the dietary shift was from bran (stage I) to PS (stage II), and to bran (stage III). In addition, the co-diet and the suitable ratio of supplemented bran were discussed based on mealworm survival and growth, and the gut microbiome of mealworms fed plastic diets and co-diets were investigated (i.e., bran, PS, PE, PS + bran, and PE + bran).

## 2. Materials and methods

### 2.1. Plastics and mealworms

The PS foam tested for biodegradation (density  $0.006 \pm 0.0003 \text{ g/cm}^3$ ) was purchased from Zhenchuang Plastic Industry Co., Ltd. (Jiangsu, China). The LDPE foam used in the experiment (density  $0.019 \pm 0.00005 \text{ g/cm}^3$ ) was obtained from Baiyangchaoqian Packaging Factory (Zhejiang, China). The mealworms (*T. molitor* L.) were purchased from Dafa Bird and Flower Market (Heilongjiang, China). The initial length of each larva was  $1.72 \pm 0.27 \text{ cm}$ . All mealworms were fed substantial edible bran purchased from Fuzhiyuan Flour Co., Ltd. (Hebei, China) for 10 days before testing, to minimize the effect of a previous larval food source at the beginning of the experiment.

### 2.2. Survival and weight of mealworms

After the pre-incubation period, 1800 mealworms were evenly divided into six groups fed the different diets, including PS, PE, PS + bran, PE + bran, bran, and starvation. The bran was spread evenly on the surface of plastic foam and the bran was supplied time once it was eaten up. All groups were incubated in the dark in food-grade polypropylene containers under  $26.5 \pm 1 \text{ }^\circ\text{C}$  and  $80 \pm 4.9\%$  relative humidity. The number of living larvae and pupae in each group were counted and recorded every 5–7 days. The whole experiment with 6 groups was performed three times in parallel and that 100 mealworms were used for each of the three replicate experiments. Another batch of 3900 mealworms was evenly divided into thirteen groups fed the co-diets with different mass ratios of bran to PS or PE, including 1:1, 3:1, 5:1, 7:1, and 9:1 (w/w), as well as bran, PS, and PE only. This batch of mealworms was used to measure survival, pupation rate, and the average weight of the mealworms. To measure the weight of mealworms, the residual plastics and frass were cleaned up first, and the mealworms were then picked out and cleaned before weighted. Breeding conditions were similar with those of the previous batch.

### 2.3. Plastic consumption rates

Another 1200 mealworms were used to examine plastic consumption rate. The larvae were evenly divided into four groups of PS, PE, PS + bran, and PE + bran diet. The remaining plastic in each group was transferred to clean containers and rinsed in 75% ethanol to clear away the frass, exuviae, and other substances every 5–7 days. The net weight of the plastic was measured after it weathered in a hood for 24 h. All tests were carried out in triplicate. Tables summarized the numbers of mealworms and replicates used in each experiment were provided in Supporting Information file (Tables S1 and S2).

### 2.4. Characterization of the frass

In total, 1500 mealworms were reared to collect frass. The larvae were divided evenly into five groups and reared with PS, PE, PS + bran, PE + bran, and bran. The bran was spread evenly on the surface of plastic foam and was supplied timely once the bran was eaten up. After a 25-day incubation period, all larvae in each group were cleaned with

compressed air and transferred to clean incubators to collect the frass. The frass was stored at  $-80^{\circ}\text{C}$  as soon as it was gathered for further characterization.

The molecular weights of the residual polymer in the frass from the PS diet group and the control PS group were quantified by gel permeation chromatography (GPC) to verify depolymerization of PS by the mealworms after ingestion. The polymers in the PS feedstock sample (1.0 g) and the frass sample (1.0 g) were extracted at room temperature with 120 mL tetrahydrofuran (THF) ( $\geq 99.9\%$ , Merck KGaA, Darmstadt, Germany) for 12 h to obtain a high extraction efficiency. The THF solution containing the residual polymer was concentrated by rotary evaporation. Then, the solution of each sample was filtered through a 0.22  $\mu\text{m}$  PVDF filter into a clean glass vial and dried overnight in a hood. The residual polymer in each sample was weighed and re-suspended in THF to form the final solution with a concentration of about 1 mg residue/mL. A triplicate analysis of molecular weight of each group using the final solution was conducted by GPC (Agilent 1100, column: PLgel 5  $\mu\text{m}$  MIXED-C, 300  $\times$  7.5 mm; Agilent Technologies, Palo Alto, CA, USA) at 30  $^{\circ}\text{C}$  with a 50  $\mu\text{L}$  injection volume. THF was used as the eluent at a flow rate of 1.0 mL/min. The molecular weights of the residual PE polymer in the frass and the control PE feedstock were quantified by high temperature gel permeation chromatography (HT-GPC). After washed and cleaned the frass samples, 200 mg frass and PE samples were extracted in 5 mL 1, 2, 4 - trichlorobenzene (1, 2, 4 TCB) for 60 min at 120  $^{\circ}\text{C}$  and then the solutions were filtered for injection. A duplicate analysis of molecular weight of each group was conducted by Agilent PL - GPC220 with column of MIX-B\* 3 (Agilent Technologies, Palo Alto, CA, USA) at 150  $^{\circ}\text{C}$  with a 200  $\mu\text{L}$  injection volume and a 0.1 mg residue/mL injection concentration. 1, 2, 4 TCB was used as the eluent at a flow rate of 1.0 mL/min.

A thermogravimetric (TG) analyzer (STA449C, NETZSCH Geratebau GmbH, Selb, Germany) was used for thermal characterization of the frass from mealworms fed the plastics (PS and PE) and the control plastics (PS and PE). The heating process was from 30° to 600°C at a rate of 10  $^{\circ}\text{C}/\text{min}$  under high-purity nitrogen.

Spectrum of FT-IR Spectrometer (PerkinElmer, Inc., Shelton, CT, USA) was used to detect the functional groups of the polymers in the frass and the plastics. During the test, potassium bromide (KBr) was first ground to the powder and placed in the infrared beam to obtain the background spectrum. Subsequently, all samples were ground to the homogeneous powders separately and mixed with the KBr powder at a ratio of 1:5 by weight. The spectra were recorded in absorbance mode and transferred to transmittance for graphing, from the wave number range of 4000–450  $\text{cm}^{-1}$  with a minimum of four scans per sample at a spectral resolution of 4  $\text{cm}^{-1}$ .

## 2.5. Gut microbiome analysis

A total of 1500 mealworms were evenly divided into five groups and fed PS, PE, PS + bran, PE + bran, or bran, respectively (with the similar feeding process as with the mealworms fed for frass characterization). After a 15-day incubation, nine mealworms were randomly picked from each group for the gut microbiome analysis. Another 300 mealworms were reared under an alternating feeding pattern. The alternating feeding pattern was designed in three stages with an operational period of 2 weeks for each stage. Stage one was the bran-fed diet stage, and the larvae from stage one were then fed PS for 2 weeks during stage two. In the third stage, larvae from stage two were fed another 2 weeks successively with bran. Nine larvae were randomly selected at the end of each feeding period (i.e. every 2 weeks) for a gut microbiome analysis. All larvae were sterilized with 75% ethanol for 1 min and then rinsed twice with sterile salt water (0.85% w/v). Then, their guts were taken out under an anatomical lens. The genomic DNA of the gut microorganisms was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals Co., Ltd., Shanghai, China). The V4 region of 16S rRNA gene was amplified using the ABI GeneAmp® 9700 PCR system and a pair of

bacterial primers to analyze the gut core microbiome: forward primer 515F (5'-GTGCCAGCMCCGCGTAA-3') and reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3').

Sequencing of the 16S rRNA gene amplicon was performed on the Ion S5 XL System (Thermo Fisher Scientific, Waltham, MA, USA). Raw reads were obtained by adjusting short reads using Cutadapt (V1.9.1, <http://cutadapt.readthedocs.io/en/stable/>) (Caporaso et al., 2010), splitting data according to the barcode, and then removing the barcode and primers from each read. Clean reads were obtained by removing chimeric sequences after alignment using the UCHIME Algorithm ([http://www.drive5.com/usearch/manual/uchime\\_algo.html](http://www.drive5.com/usearch/manual/uchime_algo.html)) (Edgar, 2013; Haas et al., 2011). Operational taxonomic units (OTUs) were determined based on a 97% identity threshold using Uparse (V7.0.1001, <http://drive5.com/uparse>) (Edgar et al., 2011). A representative sequence of each OTU was selected for taxonomic identification (0.8–1.0 threshold) using the SILVA (<https://www.arb-silva.de/>) SSUrRNA database. Alpha diversity (ACE, Chao1, Simpson, and Shannon indices) and beta diversity were calculated using Qiime software (version 1.9.1).

## 2.6. Statistical analysis

One-way ANOVA ( $n = 3$ ) with post-hoc Tukey and Tamhane's T2 tests was performed for multiple comparisons on the parameters with multiple co-diet groups. T-test (Student's and Welch's) was performed for the pairwise comparison.

## 3. Results

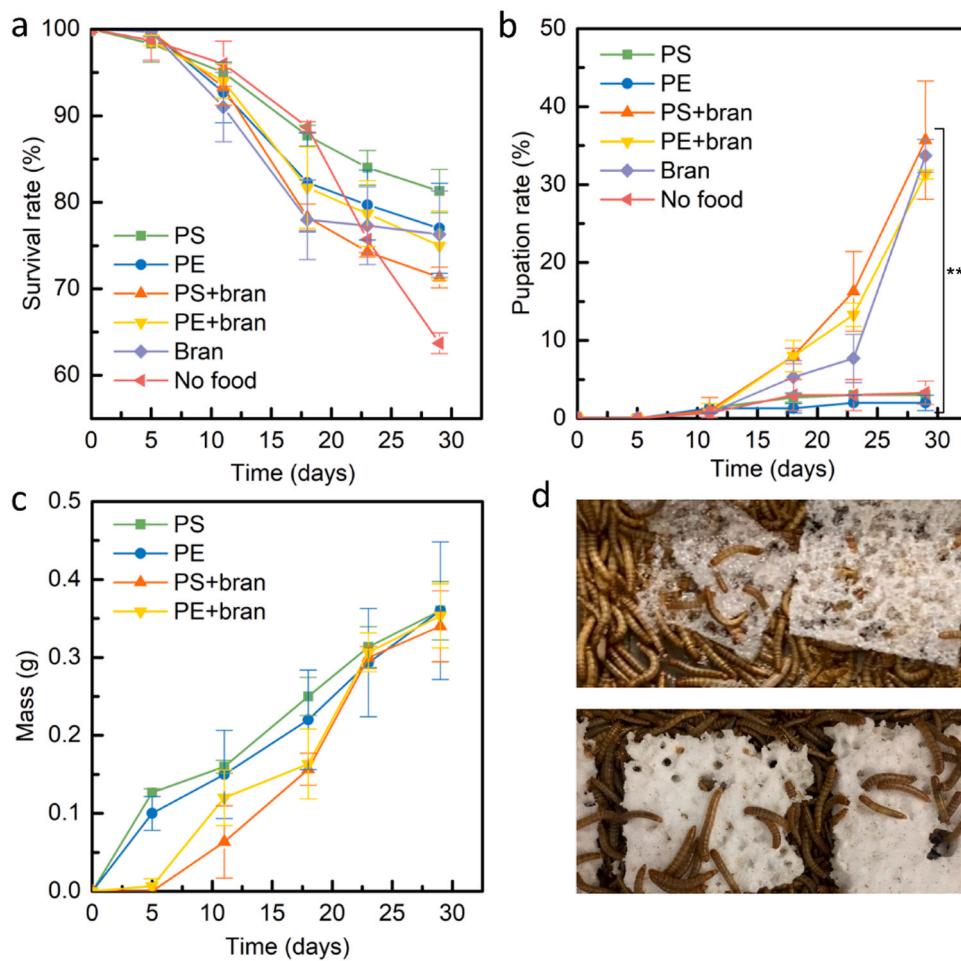
### 3.1. Growth and plastic consumption by mealworms fed different diets

The mealworms climbed on and chewed the LDPE and PS plastic foam as soon as they were fed. Over the 29-day incubation period, the survival rates (SRs) of larvae feeding on the PS, PE, and bran were 81.3  $\pm$  2.5%, 77.0  $\pm$  5.2%, and 76.3  $\pm$  5.0%, respectively (Fig. 1a). The pupation rates (PRs) of mealworms feeding on PS and PS + bran were 3.0  $\pm$  0.0% and 35.7  $\pm$  7.6%, respectively (Fig. 1b). The pupation rates of larvae eating PE and PE + bran were 2.0  $\pm$  1.0% and 31.3  $\pm$  0.6%. The pupation rate of larvae fed only plastic was lower than that of mealworms fed plastic mixed with bran. The total consumption of PS and PE increased steadily during the test period. At the end of the test, PS and PE consumptions were similar ( $0.36 \pm 0.037$  g and  $0.36 \pm 0.088$  g respectively), and the co-diet of bran with plastics also resulted in similar plastic consumption of  $0.34 \pm 0.045$  g for the PS + bran group and  $0.35 \pm 0.041$  g for the PE + bran group (Fig. 1c).

A co-diet of bran<sub>7</sub> + PE<sub>1</sub> (ratio of 7:1) exhibited the highest SR of  $90.33 \pm 4.5\%$  (Fig. 2b). The SR of the bran<sub>7</sub> + PS<sub>1</sub> co-diet group was the second for the PS groups at  $88.0 \pm 2.2\%$  which was slightly lower than that of bran-fed mealworms ( $89.33 \pm 1.9\%$ ). The bran-fed condition was the best for pupation of the mealworms (pupation rate  $31.67 \pm 6.6\%$ ), followed by the co-diet of bran<sub>7</sub> + PS<sub>1</sub> at  $27.0 \pm 4.3\%$ . The ratios of 5:1 and 7:1 for PE with bran were similar for the pupation rates ( $18.67 \pm 0.5\%$  and  $18.0 \pm 5.0\%$  respectively). The mealworms fed only bran showed the highest average weight of  $0.097 \pm 0.008$  g per worm, followed by the co-diet of bran<sub>7</sub> + PE<sub>1</sub> ( $0.084 \pm 0.005$  g per worm) (Fig. 2). The ratios of bran<sub>7</sub> + PS<sub>1</sub> co-diet and bran<sub>9</sub> + PS<sub>1</sub> co-diet were similar for the weights of the mealworms, which were  $0.0785 \pm 0.0013$  g per worm and  $0.0784 \pm 0.0055$  g per worm, respectively. The pupation rates and average weights of the mealworms fed only plastics were very low.

### 3.2. Characterization of biodegradation of the egested plastics

The GPC analysis showed an increase of number-average molecular weight ( $M_n$ ) compared the frass of PE-fed mealworm with PE feedstock sample, while weight-average molecular weight ( $M_w$ ) and size-average



**Fig. 1.** The survival and growth of mealworm, and plastic consumption by mealworms fed plastic and bran. Survival rate (a), pupation rate (b) of mealworm, and plastic consumption (c) by mealworms fed plastic and bran. Chewing and ingestion of polyethylene (upper) and polystyrene (lower) foams by the mealworm (d). One-way ANOVA,  $P < 0.01$  marked with \*\* representing the significance between groups.

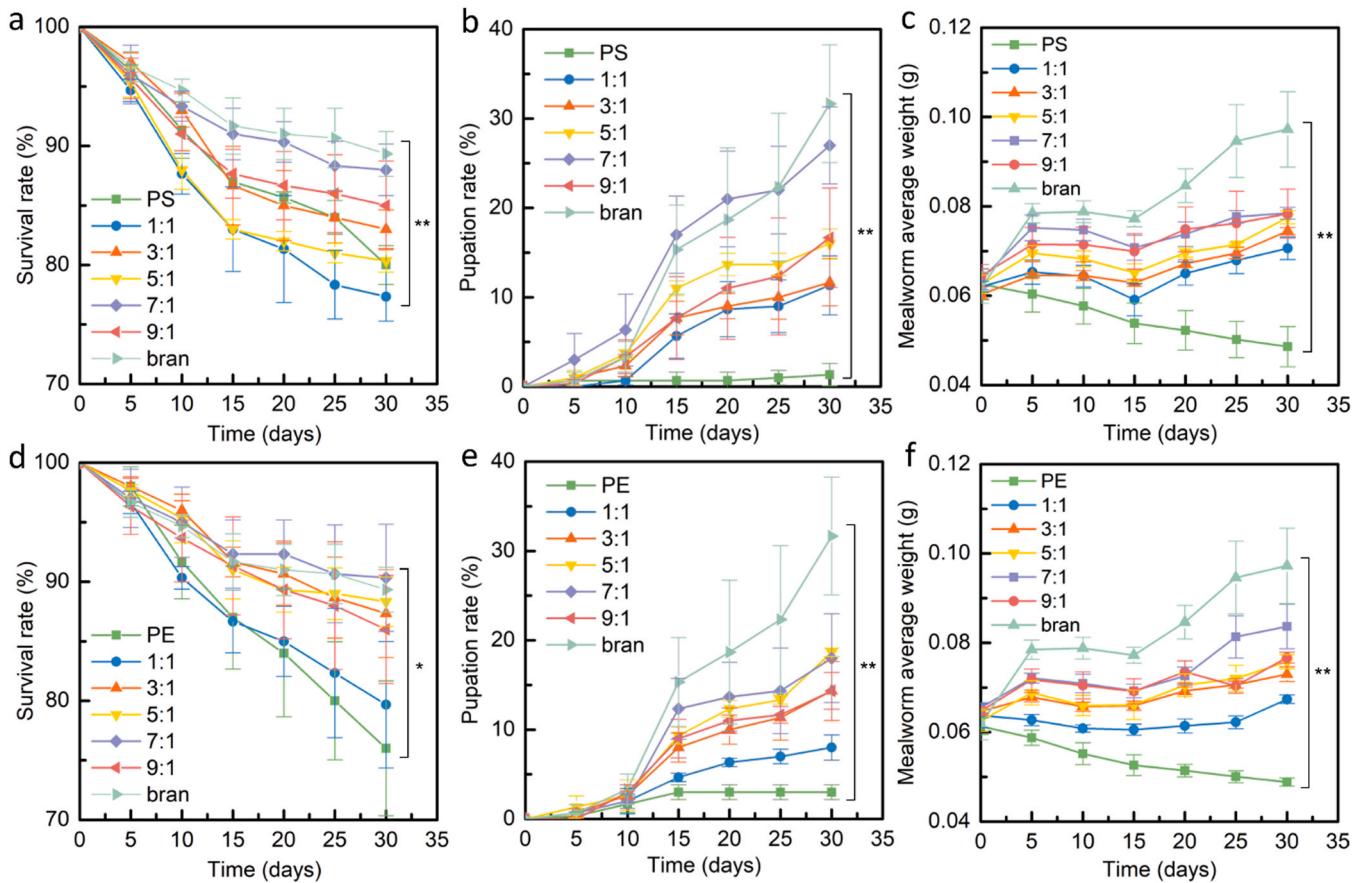
molecular weight ( $M_z$ ) of frass from PE-fed mealworm decreased compared with the PE sample from the perspective of mean value (Fig. 3f).  $M_n$  measures the average molecular weight based on the number of molecules, regardless of the size and the weight of molecules, however,  $M_w$  takes the contribution of the weight of every molecule on the average molecular weight of the materials in consideration.  $M_z$  is more sensitive to the larger molecules than the other two parameters. Therefore, the decrease of  $M_w$  and  $M_z$  for PE-fed group can also illustrate the partial depolymerization of PE polymer. All these three types of average molecular weights ( $M_n$ ,  $M_w$  and  $M_z$ ) of frass from PS-fed mealworm were decreased compared with the PS feedstock sample (Fig. 3c). The FT-IR analysis showed that new functional groups of C=O stretch ( $1700\text{ cm}^{-1}$ ) and C-O stretch ( $1050\text{--}1150\text{ cm}^{-1}$ ) were detected in the frass from the PS and PE diets (Fig. 3a and d). The peak in the range of  $2500\text{--}3500\text{ cm}^{-1}$  from the frass of mealworms fed plastics were broadened compared with that of the raw plastic samples. The FT-IR spectra of mealworm frass fed the PS/PE diet and the PS/PE + bran diet were similar with each other. Additional clues about the production of new compounds in the frass from PS and PE diets were provided by the TG analysis. Frass from both plastics lost weight in a broader range of temperatures and remained more mass after the heating process than the control plastics (Fig. 3b and e). An obvious energy change in the DTG curves of both PS and PE was observed simultaneously, but minor changes were observed in the frass from larvae fed PS and PE. These results demonstrate that PS and PE were biodegraded by the mealworms.

### 3.3. Gut core microbiomes of the mealworms fed different diets

Different diets resulted in variations in the gut microbiome of the mealworm with different diets and also within triplicate samples from the same diet. The gut microbiome of PE-fed larvae showed lower species richness based on the low ACE and Chao1 estimators compared with the other groups, as well as the numbers of OTUs and observed species (Table S3). Species richness in the gut of mealworms fed PE combined with bran increased compared with that of mealworms fed PE, which was based on the relatively higher ACE and Chao1 estimators of the PE + bran diet group. Shannon and Simpson indices represent the richness and the evenness of microbial communities. A higher community biodiversity can be concluded on the basis of a higher Shannon index or a lower Simpson index. Based on the Shannon and Simpson indices, the addition of bran did not enhance richness or evenness of the larval gut microbiome with the PS + bran co-diet.

A principal coordinates analysis (PCoA) exhibited distinctions in the core gut microbiome between the different diets (Fig. 4a). Furthermore, triplicates of each diet displayed notable variation, representing the individual variations of the mealworms under the same living conditions. Microbial community structure of the PE + bran co-diet was distinguishable from the other plastic diets based on the nonmetric multidimensional scaling (NMDS) analysis (Fig. 4b). The difference in predominant populations between different feeding diet groups were furthered exhibited in the heatmap based on weighted UniFrac distance (Fig. 4c).

Most of the gut microbes belonged to three predominant phyla of



**Fig. 2.** The survival and growth of mealworms fed different bran to plastic ratios (bran to PS/PE ratio: 0:1, 1:1, 3:1, 5:1, 7:1, 9:1, and 1:0, w/w). Survival rate of mealworm fed different ratio of bran to PS (a) and PE (d), pupation rate of mealworm fed different ratio of bran to PS (b) and PE (e), and average weight of mealworm fed different ratio of bran to PS (c) and PE (f). One-way ANOVA,  $P < 0.05$  marked with \*,  $P < 0.01$  marked with \*\*, representing the significance between groups.

*Firmicutes*, *Tenericutes*, and *Proteobacteria* despite the feeding conditions (Fig. 5a). The predominant populations of larvae fed the different diets varied widely. *Spiroplasma*, *Lactococcus*, and *Enterococcus* were predominant genera for all of the diets (Fig. 5b). *Bifidobacterium*, *Acinetobacter*, and *Streptococcus* were three genera exhibiting obviously higher relative abundances in larvae fed the PS diet than those of the other diets, in which *Bifidobacterium* and *Acinetobacter* were predominant within the PS fed group, with relative abundances of 10.09% and 4.80%, respectively. The predominant population of the gut core microbiome for (PS + bran)-fed larvae was *Pediococcus* (relative abundance 18.25%), which increased compared with the PS-fed and bran-fed larvae (relative abundance 0.18% and 0.22% respectively). Larvae fed PE had a higher relative abundance of 44.34% in the genus *Spiroplasma* than that of the bran fed group (29.08%), when the relative abundance of *Lactococcus* in the gut of larvae fed PE + bran (28.51%) was higher than that in the bran feeding diet (15.34%).

#### 3.4. Response of the mealworm gut core microbiome to a diet shift

The gut microbiome was analyzed successively at the end of each stage of an alternating feeding pattern. The alpha diversity analysis of the gut microbiome with the alternating feeding pattern indicated that the triplicate of the third stage was different and tended to be less rich and even than the first two stages (Table S4). The PCoA and NMDS analyses revealed that the gut microbiomes between different feeding stages were notably different from each other and also from the triplicate samples in the same stage (Fig. 6a and b).

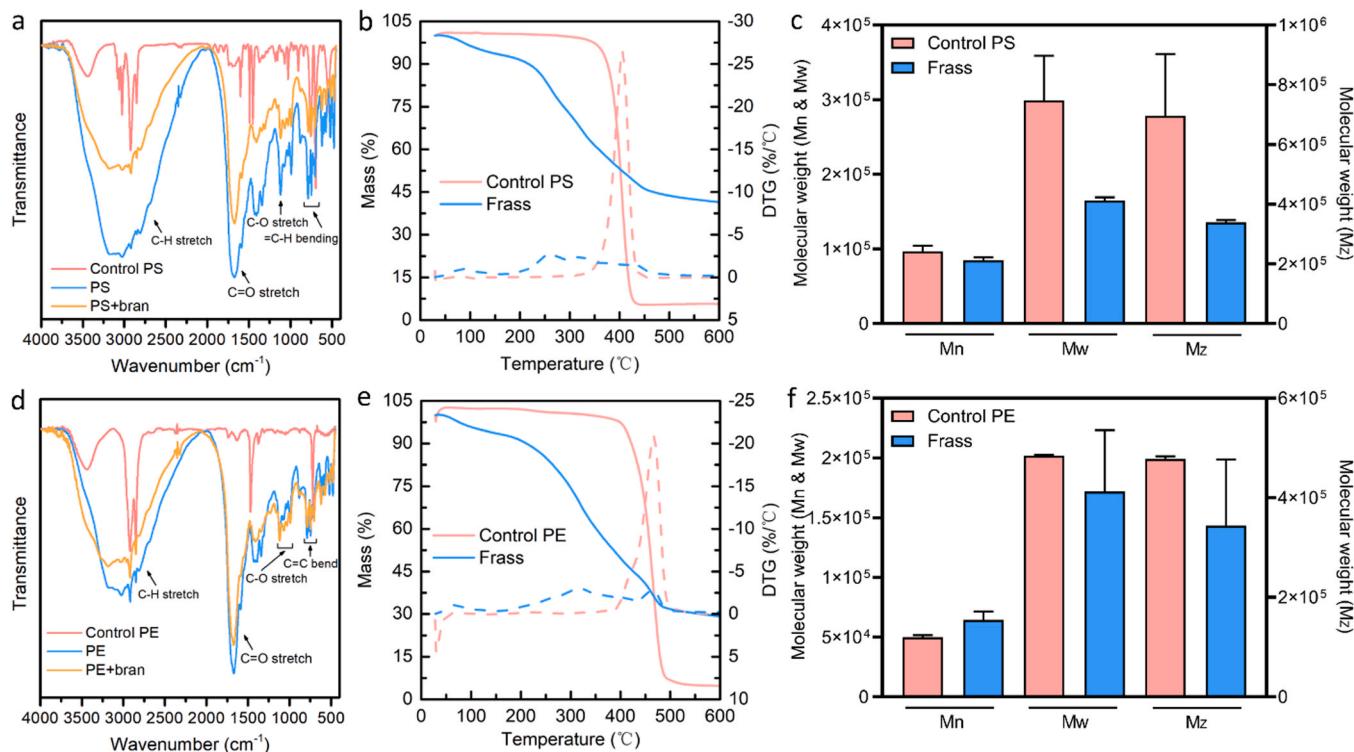
The gut microbial community structure changed with the altered feeding pattern (Fig. 7). The predominant genus of *Spiroplasma* (relative

abundance 71.98%) in the gut microbiome of mealworms fed the bran diet (stage I) increased to 82.19% after changing to the PS diet for 2 weeks (stage II). However, the superiority of *Spiroplasma* decreased and genus *Lactococcus* (relative abundance 44.40%) became the predominant genus during stage III when the diet was changed back to bran for another 2 weeks (Fig. 7b). *Enterococcus* and *Escherichia-Shigella* with relative abundances of 5.05% and 8.59% in the stage I mealworm gut decreased to 1.84% and 0.59% in the gut of stage III mealworms. The relative abundance of *Lactobacillus* in the gut of stage I mealworms was 0.32%, which increased to 6.95% in the gut of stage III mealworms.

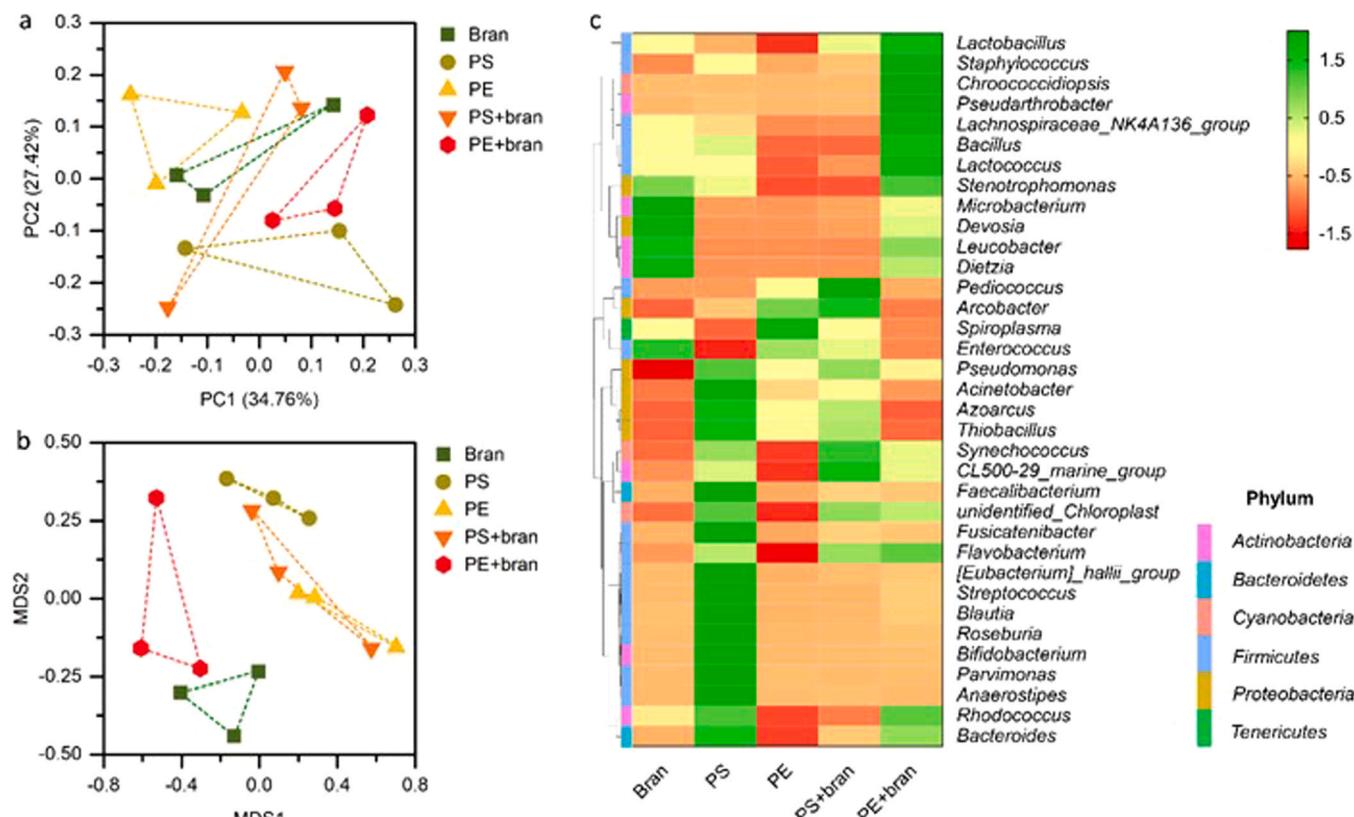
## 4. Discussion

### 4.1. Survival of mealworms and plastic consumption efficiency

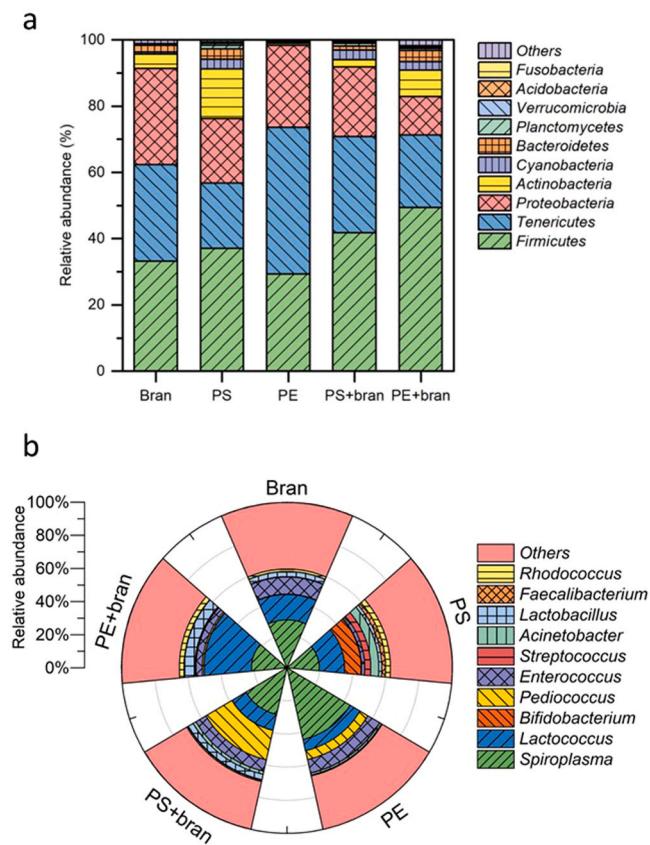
The analysis of mealworm SRs implies that neither PS nor PE had adverse effects on mealworm survival (Fig. 1a). There was no significant difference in SRs ( $P > 0.05$ ), but of significant difference in PRs ( $P < 0.01$ ) between different diet groups based on one-way ANOVA ( $n = 3$ ). The pupation rate of larvae fed the PE + bran diet increased significantly compared with those fed the PE diet ( $P < 0.01$ ). These results suggest that plastics as the only carbon source do not provide sufficient nutrients to support growth and pupation of mealworms. Therefore, extra nutrients are required if establishing a circular and continuous reactor for plastic biodegradation by mealworms. The survival rate, pupation rate and average weight of mealworm fed PS/PE with different ratios of bran exhibited significant differences between groups ( $P < 0.01$  or 0.05). The co-diet of bran<sub>7</sub>+plastic<sub>1</sub> significantly increased survival rate of mealworms compared with those fed only



**Fig. 3.** Characterizations of mealworm frass illustrating degradation of plastics. (a and d) Fourier transform infrared spectroscopy (FTIR) analysis, (b and e) thermogravimetric analysis (TG), and (c and f) gel permeation chromatography (GPC) analysis for polystyrene (PS) or polyethylene (PE) sample and frass of mealworm fed the PS or PE diet. In Fig. b and e, solid lines represent mass curve (left axis) and dashed lines represent DTG curve (right axis).



**Fig. 4.** Beta diversity analysis of mealworm gut microbiome fed the different diets. Principal coordinates analysis (a) and nonmetric multidimensional scaling (b) based on weighted UniFrac. (c) Heatmap and hierarchical cluster analysis of the predominant populations (top 35).



**Fig. 5.** Relative abundance of predominant phyla (a) and genera (b) (top 10) in the gut of mealworms fed the different diets.

plastics ( $P < 0.05$ ). The pupation rate of the bran<sub>5</sub>+plastic<sub>1</sub> co-diet group also increased compared to that of those under the plastic-only conditions ( $P < 0.05$ ). These results illustrate that the mealworm survival and growth were significantly affected by the amount of bran adding into the plastics, and the ratios of 7:1 and 5:1 (w/w) promoted the survival and growth of plastic-feeding mealworms, respectively. Consumptions of PS and PE were similar under the plastic-only nutrient conditions, suggesting that the mealworms exhibited similar capacities to ingest and degrade PS and PE in this experiment (Fig. 2). The plastic consumption rates of the co-diet groups were slower than those of the plastic-only groups at the first 5 days of experiment, possibly because the mealworms had not adapted to the recalcitrant plastic as a diet, and preferred bran when it was provided. Plastic consumption increased rapidly after the first 5 days of experiment, and reached a level similar to the plastics-only groups.

#### 4.2. Biodegradation of plastics by the mealworms

PS and PE plastics were confirmed to be degraded by the guts of mealworms based on testing the compound structure and characteristics of the frass from the mealworms. The GPC analysis revealed decreases in both  $M_n$  and  $M_w$  as well as  $M_z$  from PS to the frass of the mealworms fed PS, directly showing that PS was biodegraded via broad depolymerization (Fig. 3c), which was observed previously (Yang et al., 2015a; Yang et al., 2018a, 2018b; Yang, L. et al., 2021). The frass of mealworm fed PE showed an increase of  $M_n$  and decreases of  $M_w$  and  $M_z$  compare to the PE sample, which imply a limited extent depolymerization process differ from the depolymerization pattern of PS groups (Fig. 3f). Both broad and limited extent depolymerization of PE foams by mealworms were observed during previous PE degradation tests (Brandon et al., 2018; Yang, L. et al., 2021). The different depolymerization patterns could be mainly due to the difference of PE components used for the foams because

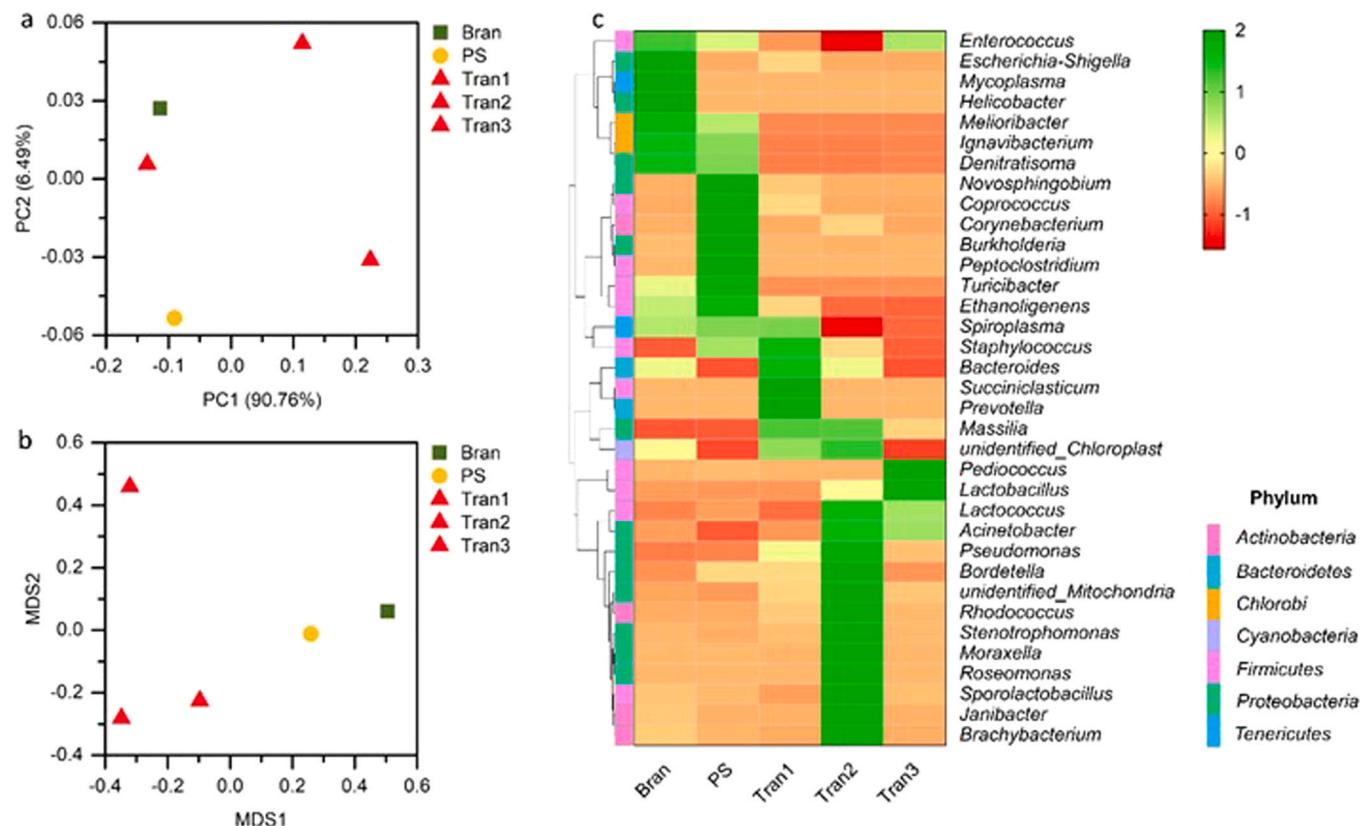
commercial LDPE products (foam, film, etc.) are mixtures of majority of LDPE with minority of HDPE and LLDPE. Previous studies also reported that PS can be chemically modified by mealworms (Yang et al., 2015a, 2015b; Yang et al., 2018a, 2018b). The FT-IR analysis revealed changes in the functional groups between the plastic samples and the frass of the mealworms fed the plastics (Fig. 3a and d). New functional groups, such as C=O stretch and C-O stretch, appeared in the frass of mealworms fed the PS and the PE diets compared with the bran control, indicating that oxygen was incorporated into the polymers during the biodegradation process in the mealworm gut. The frass curves were broadened at 2500–3500 cm<sup>-1</sup> compared to those of the plastic sample controls, representing an increase in frass hydrophilicity. Therefore, these observations confirmed the compound changes in the frass of the mealworms. The FT-IR spectra of the frass from mealworms fed the plastics and the co-diet of plastics + bran exhibited similar curves for both PS and PE conditions. This observation suggests that the biodegradation process of plastics in the gut of mealworms should be similar despite the bran supplement.

The TG analysis provided evidence that new compounds were produced in the frass of mealworms fed plastics (Fig. 3b and e). The weight losses of the PS and the PE samples (control samples) occurred in a narrower temperature range compared with those of the PS-fed and the PE-fed mealworm frass, respectively. The weight losses of the PS and the PE samples were greater than those of the PS-fed and the PE-fed mealworms frass, respectively. These findings suggest that new compounds were formed in the frass compared with the original plastics, and that some of them decomposed at a lower temperature than the original molecules, while others might only decompose at a higher temperature.

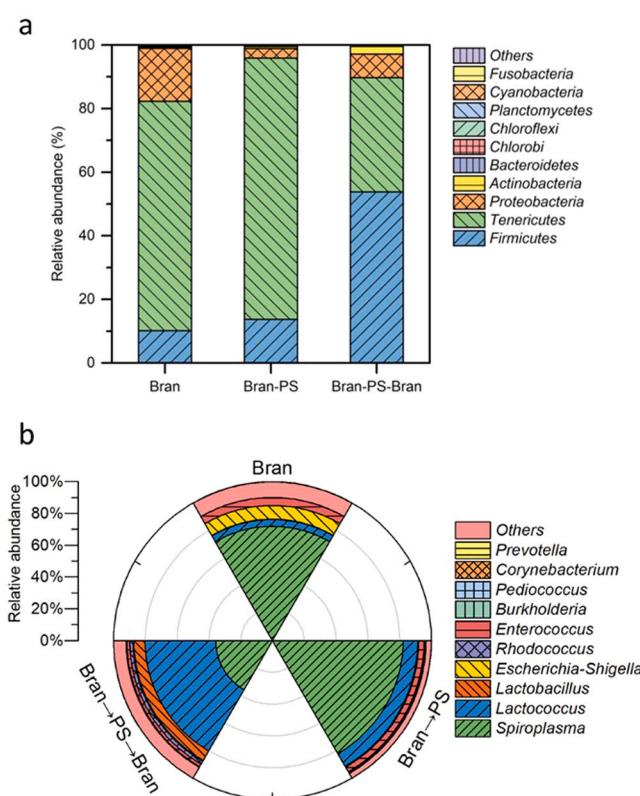
#### 4.3. Core gut microbiome of the mealworms was shaped by the plastic diets and the co-diets

The alpha diversity exhibited no significant difference between different diet groups (one-way ANOVA,  $P > 0.05$ ,  $n = 3$ ), suggesting that eating the plastics and co-diets did not significantly affect species diversity in the gut of the mealworm. However, although diversity did not significantly change with the feeding patterns, the predominant populations and their relative abundance varied (Fig. 4). *Spiroplasma*, *Lactococcus*, and *Enterococcus* were predominant in the mealworms in all five diet groups, suggesting that they might be indispensable for digestion and survival of the mealworms (Fig. 5b). A previous research also reported a high abundance of *Spiroplasma* in the gut microbiome (Koch and Schmid-Hempel, 2011), which is generally recognized as pathogenic to insects, plants, and vertebrates (Bové, 1997). However, *Spiroplasma* in the mealworm gut is not harmful (Jung et al., 2014). In addition, *Lactococcus* and *Enterococcus* were common genera in the mealworm gut microbiome, of which *Lactococcus* was present in every part of the gut while *Enterococcus* was absent in the foregut and anterior midgut (Engel and Moran, 2013). In fact, understanding the approximate parasitic sites of different bacteria and their metabolic pathways is helpful to infer possible degradation pathways of the plastics in the mealworm gut.

Supplementing with bran shifted the gut microbiome of the mealworms fed only plastics. *Pediococcus* was observed at relative abundances of only 0.22% and 0.18% in the normal food (bran-fed) and the PS-fed groups, respectively, which increased to 18.25% in the PS + bran diet group. The relative abundance of *Lactococcus* reached 28.51% in the PE + bran diet group, which was higher than that of the bran diet (15.34%) and PE diet (6.91%) groups (Fig. 5). *Pediococcus* and *Lactococcus* are lactic acid bacteria, which may have contributed to adjusting and maintaining the health of the gut microbiome environment. *Lactobacillus* and *Mucispirillum* were significantly associated with the PE + bran diet compared to the bran diet and the PE diet ( $P < 0.05$ ) (Table 1). These results suggest that the response of mealworm gut microbiome to the bran and plastic mixture was distinguished from either bran or plastics alone.



**Fig. 6.** Beta diversity analysis of the mealworm gut microbiome fed an alternating feeding pattern. Principal coordinates analysis (a) and nonmetric multidimensional scaling (b) based on weighted UniFrac. (c) Heatmap and hierarchical cluster analysis of the predominant populations (top 35).



**Fig. 7.** Relative abundance (top 10) of the mealworm gut microbiome fed an alternating feeding pattern at the phylum (a) and genus levels (b).

*Brooklawnia* was significantly associated with PS diet compared to the bran diet (Table 1), which might play a key role in hydrolysis and acidogenesis during anaerobic process (Bae et al., 2006; Kim et al., 2017). A previous report showed that *Brooklawnia* as a facultatively anaerobic genus was the predominant functional population during the bioprocess of hydrolyzed polyacrylamide degradation (Song et al., 2018). Besides, *Bifidobacterium*, *Acinetobacter*, and *Streptococcus* were the predominant populations in gut microbiome of mealworms fed the PS diet, but in relatively low abundance under the other diet conditions (Fig. 5), among which *Acinetobacter* degrades up to 40% of the crude oil in 28 days in a previous research (Obuekwe et al., 2009). Therefore, these bacteria were deduced to be involved in the degradation of PS.

In comparison of the PS-fed and the PE-fed diets, six more genera were significantly associated with the PS diet including *Weissella*, *Delftia*, *Prevotella*, *Blastococcus*, *Legionella*, *Probacacter*, while only *Enterococcus* was associated with the PE diet (Table 1). The most abundant genus among these genera was *Delftia*, a strictly aerobic chemoorganotrophic bacterium (Horowitz et al., 1990). Previous studies showed that *Delftia* sp. could utilize different aromatic compounds as sole carbon and energy sources. For example, *Delftia tsuruhatensis* degrades terephthalate under aerobic conditions (Shigematsu et al., 2003) and *Delftia* sp. AN3 is capable of degrading aniline (Zhang et al., 2008). Therefore, a specific *Delftia* sp. may participate in the degradation process of the benzene ring in PS. The only genus associated with PE degradation was *Enterococcus*, a Gram-positive facultative anaerobe (Murray, 1990). One strain belonging to this genus was isolated in a previous study as a PE-degrading bacterium (Albertsson et al., 1987).

The gut microbial community structure of the triplicate samples of each feeding diet exhibited distinction, representing the individual variations had an effect of shaping the gut microbiome of mealworm. In our study, 9 mealworms were chosen randomly from each of the triplicate samples of the same feeding diet to weaken the influence of

**Table 1**

Genera with significantly different relative abundances ( $P < 0.05$ ) between two groups based on Welch's *t*-test. (Mean value 1 represents the mean value of relative abundance for the former compared group and Mean value 2 represents the mean value of the latter compared group. B, S, E, BS, and BE represents the feeding diets of bran, PS, PE, PS + bran, PE + bran, respectively. The genera chosen for comparing S vs. BS and E vs. BE were based on the results of B vs. BS and B vs. BE, respectively.).

Compared groups	Taxa	Mean value 1	Mean value 2	P-value
B vs. S	<i>Atopostipes</i>	3.48E-04	1.20E-04	3.97E-02
B vs. S	<i>Brooklawnia</i>	6.33E-05	1.39E-04	1.12E-02
B vs. E	<i>Rhodococcus</i>	1.43E-02	2.48E-03	6.93E-03
B vs. E	<i>Stenotrophomonas</i>	6.16E-03	1.01E-03	1.04E-02
B vs. E	<i>Ilumatobacter</i>	8.29E-04	1.65E-04	1.26E-02
B vs. E	<i>Flavobacterium</i>	1.12E-03	2.60E-04	4.43E-02
B vs. E	<i>Achromobacter</i>	1.47E-03	1.33E-04	3.58E-02
B vs. E	<i>Paenarthrobacter</i>	9.49E-05	0.00E+00	4.94E-02
B vs. E	<i>Atopostipes</i>	3.48E-04	2.53E-05	1.23E-02
B vs. E	<i>Clostridium</i>	1.20E-04	6.33E-06	3.92E-02
B vs. BS	<i>Stenotrophomonas</i>	6.16E-03	1.11E-03	9.86E-03
B vs. BS	<i>Achromobacter</i>	1.47E-03	2.53E-04	3.04E-02
B vs. BE	<i>Lactobacillus</i>	3.04E-02	6.32E-02	2.39E-02
B vs. BE	<i>Mucispirillum</i>	0.00E+00	2.34E-04	2.60E-02
S vs. E	<i>Enterococcus</i>	1.97E-02	8.27E-02	2.18E-02
S vs. E	<i>Weissella</i>	1.33E-04	1.27E-05	1.09E-02
S vs. E	<i>Delftia</i>	1.05E-03	1.46E-04	1.16E-03
S vs. E	<i>Prevotella</i>	4.24E-04	2.53E-05	2.84E-02
S vs. E	<i>Blastococcus</i>	1.58E-04	5.06E-05	3.97E-02
S vs. E	<i>Legionella</i>	1.08E-04	1.90E-05	4.23E-02
S vs. E	<i>Procabacter</i>	3.23E-04	1.27E-05	2.01E-02
E vs. BE	<i>Lactobacillus</i>	1.93E-03	6.32E-02	7.44E-03
E vs. BE	<i>Mucispirillum</i>	3.16E-05	2.34E-04	1.53E-02

individual variations (Brandon et al., 2018). Besides, the microbial community analysis was performed based on the triplicate samples for each feeding condition in the statistical algorithms, and several genera exhibited significant differences between certain feeding diets ( $P$  values in Table 1). Therefore, the distinction of different groups observed was believed could be ascribed to corresponding feeding conditions. However, the individual variations of mealworms should be a nonnegligible factor in the research of plastic biodegradation by mealworm and its gut microbes. Plastic biodegradation and gut microbiome of individual mealworm under same feeding condition should be further evaluated in the future.

#### 4.4. Response of the mealworm gut core microbiome to a diet shift

The mealworm gut core microbiome analysis with an alternating feeding pattern showed that the relative abundance of some

predominant genera changed considerably at the end of each feeding stage (Fig. 7). The predominant genus of *Spiroplasma* during the first stage (bran diet) was replaced by *Lactococcus* at the end of the third stage (bran – PS – bran), though both the first and third stages were bran-fed diets. *Enterococcus* and *Escherichia-Shigella* also decreased while *Lactobacillus* and *Lactococcus* increased during the third stage compared to the first stage based on their relative abundance. *Lactobacillus* and *Lactococcus* are common lactic acid bacteria, which typically are considered to help to maintain a stable gut environment, improve the distribution of gut microbes and prevent the colonization of harmful bacteria (Fang Yan, 2011). The continuing increase of these bacteria during the process of alternating the diets exhibited that the PS diet might have not caused harmful impact on the gut microbiome of mealworm. In addition, the distinction between the gut microbial structure of mealworm in stage I and III suggested that the shaping of gut core microbiome under a specific diet was influenced by both the current diet and the previous diets if applicable.

The microbial community assembly is driven by the combination of stochastic and deterministic processes. Stochastic processes are typically dominant in the initial establishment of a microbial community, and deterministic processes then govern the microbial community succession as selection strength increases (Dini-Andreote et al., 2015; Yuan et al., 2019). Under the same nutrient condition, the stage III gut microbiome was distinguished from that of stage I, suggesting that gut community assembly might be dominated by stochastic processes. A strong disturbance in the environment could reset the microbial community assembly, which would break the stability of the previous community structure (Ferrenberg et al., 2013). In some ecosystems, the community is reassembled and governed by stochastic processes after a particular disturbance (Dini-Andreote et al., 2015; Ferrenberg et al., 2013). Here, the sudden shift in the mealworm diet may have disturbed the intestinal ecosystem, which would break the stability of the microbial community structure shaped by the previous diet. Then, the gut microbial community might be reestablished and governed by stochastic processes, resulting in the distinction of the microbial community structure between stages I and III. Additionally, as mentioned above, individual differences may have existed among mealworms, which also contributed to the deviation between the gut microbiomes under the same nutrient diets. The dominance of stochastic processes during microbial assembly after a disturbance in the gut environment might also be an explanation for individual variations. To date, the research on biodegradation of plastics has been focused on the activities of large group but not individuals. Further related research should be performed to explain the interaction between gut microbial community assembly and individual variations, which could help to explore the mechanisms for the plastic degradation by mealworms and its gut microbiome.

The exploration of the gut microbiome of mealworm shaping by the alternating feeding pattern was the highlight section in our research compared with the previous related researches (Brandon et al., 2018; Yang et al., 2018b). It could help in understanding the gut microbiome succession and the response of the gut microbiome to different feeding processes, and further provide support in exploring the mechanism of mealworm plastic biodegradation. The enhancement of bran as co-diet with different ratios to the plastic-fed mealworm on its plastic biodegradation efficiency and the effect of co-diet on the mealworm gut microbiome in this study seemed similar with a few previous researches (Brandon et al., 2018; Yang et al., 2018b). However, some novel observations were displayed and new focuses were discussed in this research. The individual variations of mealworms were observed and focused, which caused the consideration that the plastic biodegradation by the gut microbes of mealworm may be individualized. Therefore, whether or not that the plastic-degrading bacteria could be enriched in the gut of every individual mealworm is worthy of further exploration to comprehensively understand the mechanism of mealworm plastic biodegradation. In addition, the emulsifying factors were secreted by the mealworm in their gut, which assisted the gut microbes to facilitate

plastic biodegradation (Brandon et al., 2021). Hence, plastic biodegradation by the emulsifying factors and gut microbes of the mealworms with shifting diets should be evaluated in the future.

## 5. Conclusion

This study investigated biodegradation of PS and PE with supplementary bran as co-diets by mealworms, which laid the foundations for the construction of efficient plastic-biodegrading reactor with mealworm and its gut microbes. A bran and plastic mass ratio of 7:1 (w/w) facilitated the growth of plastic-feeding mealworms. The gut microbiomes of mealworms fed co-diets exhibited substantially distinction from those of bran-fed and plastics-fed groups, suggesting that the effect of bran and plastics mixture on the community assembly of gut microbes was distinguished from either bran or plastics alone. The analysis of the larval gut microbial community with an alternating feeding pattern at the end of the third stage revealed shifts from the first two stages, which may have been attributed to stochastic processes during microbial succession, indicating that the gut microbiome can be shaped by the feeding process and previous domestication of larvae by the diet.

## CRediT authorship contribution statement

**Yu Lou:** Investigation, Data curation, Writing - original draft, Writing - review & editing. **Yiran Li:** Investigation, Data curation, Writing - original draft. **Baiyun Lu:** Investigation, Data curation. **Qiang Liu:** Investigation, Data curation. **Shan-Shan Yang:** Data curation, Formal analysis. **Bingfeng Liu:** Data curation, Formal analysis. **Nanqi Ren:** Data curation, Formal analysis. **Wei-Min Wu:** Data curation, Formal analysis, Writing - review & editing. **Defeng Xing:** Data curation, Formal analysis, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition, Supervision.

## 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.

## Acknowledgments

The authors appreciate the suggestions and support by Professor Craig S. Criddle, Stanford University. This study was supported by the National Natural Science Foundation of China (No. 31870114), the State Key Laboratory of Urban Water Resource and Environment (Harbin Institute of Technology) (No. 2019DX02), and the Heilongjiang Touyan Innovation Team Program (HIT-SE-02-02).

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2021.126222](https://doi.org/10.1016/j.jhazmat.2021.126222).

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