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Short communication

Biodegradation and mineralization of polystyrene by plastic-eating superworms *Zophobas atratus*

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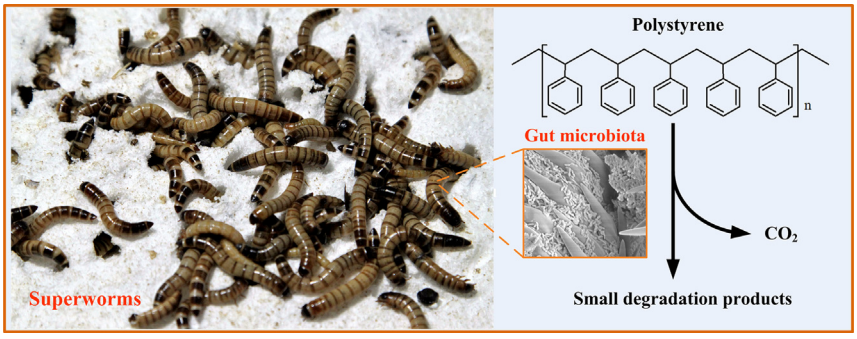
HIGHLIGHTS

- Superworms can eat and live well with Styrofoam as sole diet.
- Depolymerization of ingested Styrofoam occurred within the superworms' guts.
- Up to 36.7% of ingested Styrofoam was mineralized into CO_2.
- Gut microbiota plays a key role in the biodegradation of PS within the guts.

GRAPHICAL ABSTRACT



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ABSTRACT

Polystyrene (PS) is one of the major plastic debris accumulated in environment. Previously, we reported that mealworm (*Tenebrio molitor*) was capable of degrading and mineralizing Styrofoam (PS foam). This finding arouses our curiosity to explore whether more other insect species have the same capability as mealworms. Here, an insect larva, superworm (*Zophobas atratus*), was newly proven to be capable of eating, degrading and mineralizing PS. Superworms could live with Styrofoam as sole diet as well as those fed with a normal diet (bran) over a 28-day period. The average consumption rate of Styrofoam for each superworm was estimated at 0.58 mg/d that was 4 times more than that of mealworm. Analyses of frass, using gel permeation chromatography (GPC), solid-state ^{13}C cross-polarization/magic angle spinning nuclear magnetic resonance (CP/MAS NMR) spectroscopy, and thermogravimetric interfaced with Fourier transform infrared (TG-FTIR) spectroscopy, demonstrated that the depolymerization of long-chain PS molecules and the formation of low molecular-weight products occurred in the larval gut. A respirometry test showed that up to 36.7% of the ingested Styrofoam carbon was converted into CO_2 during a 16-day test period. The PS-degrading capability of superworm was inhibited by the antibiotic suppression of gut microbiota, indicating that gut microbiota contributed to PS degradation. This new finding extends the PS-degrading insects beyond the species within the *Tenebrio* genus and indicates that the gut microbiota of superworm would be a novel bioresource for pursuit of plastic-degrading enzymes.

1. Introduction

Polystyrene (PS), a common petroleum-based plastic made from the polymerization of styrene monomer, is used for a wide range of packaging and building constructions since its first commercial production in 1930. Nowadays, the annual production of

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PS reaches approximately 33 million tons, accounting for about 7% of the total global plastic production (Farrelly and Shaw, 2017). The increasing plastic consumption has inevitably brought about a large amount of plastic wastes. Most of PS wastes are discarded without recycling, and have become one of the major plastic debris accumulated in the environment (Jambeck et al., 2015). How to deal with the plastic wastes and remediate the plastic pollution has become a serious environmental issue within the recent years (Yang et al., 2015a,b).

PS is generally considered to be resistant to biodegradation because of its recalcitrant macromolecular structure. Although a few studies have reported that PS could be biodegraded and mineralized by mixed microbial cultures or isolates from various sources such as soil, garbage, and sewage sludge, the reported biodegradation efficiency of PS was still quite low (Guillet et al., 1974; Sielicki et al., 1978; Kaplan et al., 1979; Mor et al., 2008; Atiq et al., 2010). Recently, our study firstly reported that mealworms (larvae of *Tenebrio molitor*) could chew and eat Styrofoam (trade name of PS foam) as sole food, and rapidly degrade and mineralize the ingested Styrofoam after passage through the intestinal tract (Yang et al., 2015a). Furthermore, we demonstrated that the gut microbial symbiont plays an important role in the biodegradation of ingested Styrofoam in the gut (Yang et al., 2015b). These findings don't only open a new way for harnessing mealworms to degrade more other types of plastic wastes such as polyethylene (Brandon et al., 2018) or rubber (Aboelkheir et al., 2019), but also provide inspirations for exploring more other insect species for biodegradation of plastic wastes (Bombelli et al., 2017; Chalup et al., 2018; Kundungal et al., 2019; Peng et al., 2019).

Superworms, the larvae of *Zophobas atratus*, is another usual feed additive for pets due to its high protein and fat content, as well as the mealworms (Tschinkel, 1984). Based on the analysis of DNA barcode of COI gene (cytochrome *c* oxidase subunit I), the average genetic divergence between *Z. atratus* and *T. molitor* is 20.9%, although both of them belong to the family of *Tenebrionidae* (Park et al., 2013). The length of superworms is 3 to 6 cm, which is four times as long as mealworms. According to literature and our observations, superworms also can chew and eat Styrofoam as sole diet (Miao and Zhang, 2010). However, it remains unknown whether superworms have the same capability as mealworms to degrade and mineralize the ingested Styrofoam after passage through the gut system.

Therefore, the object of this study was to investigate the potential of superworms to degrade and mineralize Styrofoam when they were fed with Styrofoam as sole diet. According to the previously established protocols, (i) to determine whether the chemical structure and compositions of the ingested Styrofoam have changed after passage through the gut, frass of Styrofoam-eating superworms was collected and characterized by GPC, solid-state ^{13}C CP/MAS NMR and TG-FTIR; (ii) to work out the conversion efficiency of the ingested Styrofoam to CO_2, a series of respirometry tests were performed; moreover, (iii) to uncover the role of gut microbial symbiont in biodegradation of PS, antibiotic suppression treatment assays were carried out.

2. Materials and methods

2.1. Test materials

Superworms (growth age at 3–4 instars) for all tests were purchased from Daxing Insect Breeding Plant, Beijing, China. The Styrofoam (PS foam) feedstock was obtained from SINOPEC Beijing Yanshan Company, China. Its chemical composition has been identified in previous study (Yang et al., 2015a). No catalysts and additives were added as per the manufacture standard in China (QB/T 4009–2010). The number-average molecular weight (M_n) and weight-average molecular weight (M_w) is 147,000 and 274,000, respectively.

2.2. Feeding tests

A group of superworms (300 as a group) were reared on Styrofoam block (6.0 g) as sole diet in a polypropylene plastic container (L × W × H = 35 × 24 × 7 cm). As a control, other group of superworms (300 as a group) were reared on normal diet of wheat bran. Both the Styrofoam-feeding-group and the control group were prepared in triplicate (n = 3). The mass loss of the Styrofoam block caused by superworms' activities and the number of alive superworms was measured periodically. The survival rate of superworms groups fed with Styrofoam was compared to that fed with bran using a t test. All containers were maintained in the climatic chamber (RQH-250, Shanghai) under the controlled condition i.e. 25 ± 1 °C, 80 ± 2% humidity, 8:16 (L:D) photoperiod, for a period of 28 days.

2.3. Collection and characterization of frass

After a 28-day period, the Styrofoam-feeding superworms were transferred to a clean container for the collection of frass every 12 h. In this way, the carryover of un-ingested Styrofoam morsels in the frass could be avoided. The collected frass were immediately stored in liquid nitrogen for further analysis.

The molecular weight of Styrofoam or the degradation products in frass was determined using gel permeation chromatography (GPC, Alliance V2000, Waters, Milford, MA). Approximately 1.0 g of fresh frass was extracted with 150 mL of tetrahydrofuran (THF) as the solvent in a Soxhlet extractor at 90 °C for 12 h. Then, the extracted solution was concentrated to 5 mL. The injection volume was 50 μL each time. THF was used as an eluent at a flow rate of 1.0 mL/min at 40 °C.

The native compositions of Styrofoam and frass were analyzed by a solid-state ^{13}C nuclear magnetic resonance (NMR) spectrometer (AVANCE III 400, Bruker, Billerica, MA) with the techniques of proton-carbon cross-polarization/magic angle spinning (CP/MAS) at ambient temperature. The operational parameters were 1.5 ms contact time, 4 s recycle delay, 0.013 s acquisition time, 4 μs 90° pulse, and 5 kHz MAS spin.

The thermal characterization of Styrofoam and frass (ca. 5 mg) were performed using a thermogravimetric (TG) analyzer (TGA-209F1, NETZSCH, Selb, Germany) interfaced with Fourier transform infrared spectroscopy (FTIR, Nicolet Magna IR-8700, Thermo Scientific, Waltham, MA). Samples were vaporized at a heating rate of 20 °C/min from ambient temperature to 650 °C under high-purity nitrogen (99.999%) at a flow rate of 10 mL/min. The differential thermogravimetric (DTG) curves, representing the rate of weight loss as a function of temperature during thermal decomposition, was estimated according to the TG curves.

2.4. Respirometry test

A group of 30 superworms fed with Styrofoam as a sole diet were reared in a glass jars incubator (500 mL, n = 3). Other starving group of 30 superworms unfed with food were also reared in a glass jars incubator (500 mL, n = 3). A lifeless control only with Styrofoam (n = 3) was used to ensure that there no CO_2 in the inlet-air. The incubators were sealed with rubber stoppers. Compressed air passed through two CO_2 pre-trappers (2 M NaOH, 250 mL) in series to remove CO_2 from the air, moisturized and then entered the incubator. The off-air passed through another two post-CO_2 trappers (2 M NaOH, 250 mL) in series to collect CO_2 produced from the incubator. The NaOH solution in the CO_2 trappers

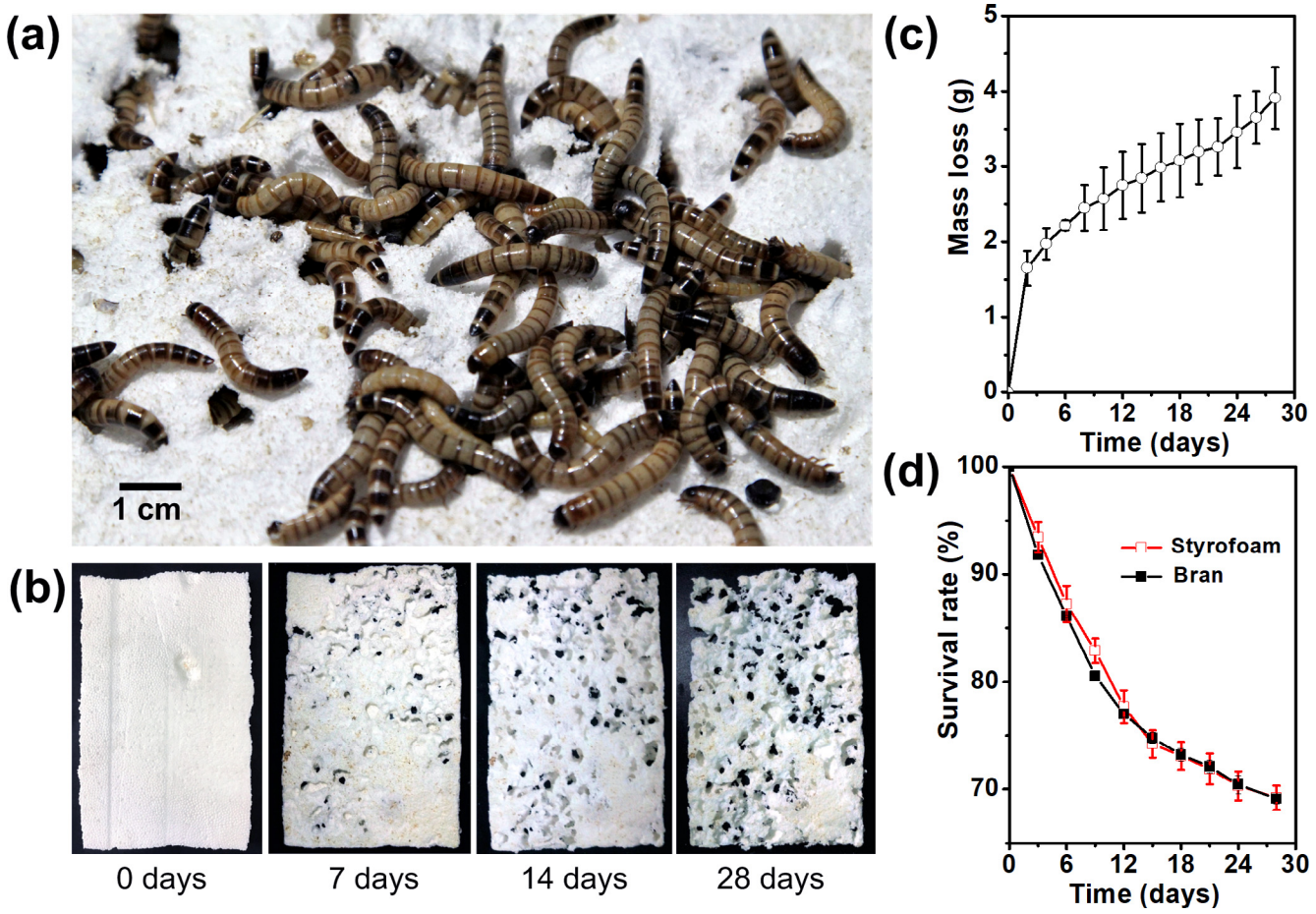

Fig. 1. Styrofoam-eating activities of superworms (*Zophobas atratus*). (a) Superworms like to eat and penetrate Styrofoam, which is also showed in SI Movie. (b) Increasing hollows in the Styrofoam block caused by the superworms' activities with incubation time. (c) Consumption of Styrofoam by a group of superworms and (d) survival rate of Styrofoam-eating and normal diet (bran)-eating superworms over a 28-day period [mean ± standard deviation (SD), n = 3 groups, 300 worms as a group].

was replaced every day. The CO_2 collected in NaOH solutions was precipitated with $BaCl_2$ to $BaCO_3$, which was measured after being dried to a constant weight. The measured dry weight of $BaCO_3$ was used for the calculation of CO_2 trapped.

A series of carbon mass balance batch experiments were carried out over a number of incubation periods for 4, 8, 12 and 16 days, respectively. Three parallel incubators were prepared for each incubation period. At the end of each incubation period, three parallel incubators were sacrificed and the total CO_2 production, the mass of ingested Styrofoam and egested frass were determined. The carbon contents of Styrofoam and frass were determined using Elemental Analyzer (Vario EL, USA). The conversion of ingested Styrofoam to CO_2 and frass was estimated using the detailed procedures described in the previous study (Yang et al., 2015a).

2.5. Antibiotic suppression treatment assay

A group of superworms (300 as a group, n = 3) were fed with the antibiotic diet (60 mg/g of bran food; weight ratio, gentamicin:rifampicin:streptomycin = 3:2:6) for 8 days, whereas the other control group were fed with normal bran without antibiotic. At 0, 2, 4, 6 and 8 days, 10 superworms were randomly selected from each group for analysis of the number of active gut bacteria by the series dilution method of plate counting. When the number of active gut bacteria became approximately zero, the remaining antibiotic-treated superworms were subsequently fed with Styrofoam and their frass were collected for the analysis of molecular weight by GPC. The morphology of the microorganisms dwelling on the gut wall of superworms was observed by an environment scanning electron microscope (ESEM, Quanta FEG250, FEI Company, Hillsboro, OR).

3. Results and discussions

3.1. Styrofoam-eating activities

When superworms were fed with Styrofoam as sole diet, they immediately began to eat and penetrate the Styrofoam blocks (Fig. 1a and SI Movie). Hollows appeared in the Styrofoam blocks after 1 h and increased with the time of incubation (Fig. 1b). Based on the mass loss of Styrofoam block caused by superworms' eating-activities, the consumption of Styrofoam by superworms could be estimated. Over the 28-day test period, a group of superworms can eat 3.9 ± 0.4 g of Styrofoam (Initial number is 300, n = 3 groups) (Fig. 1c). We set the median active superworms (about 240) during 28 days as the base number for the calculation of average consumption of Styrofoam per worm (Fig. 1d). The average Styrofoam consumption rate could be calculated as approximately 0.58 mg/d per worm, which was 4 times more than that of mealworms (0.12 mg/d per mealworm) (Yang et al., 2015a).

As shown in Fig. 1d, the survival rate (SR) of Styrofoam-eating superworms was not significantly different from that of normal diet (bran)-eating superworms (300 worms as a group, n = 3 groups; t test, $p > 0.05$). After 28 days, superworms continued to

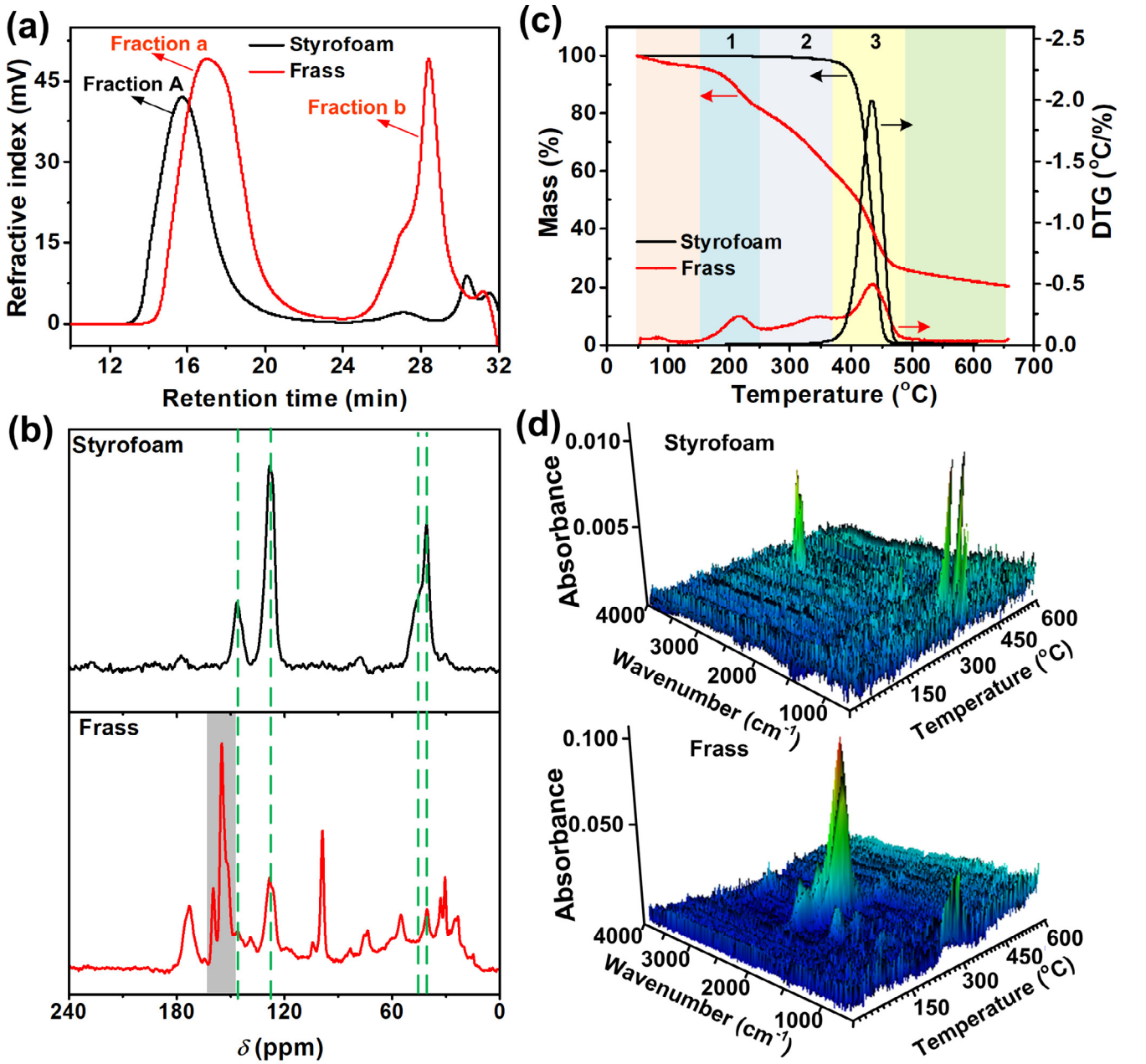

Fig. 2. Chemical analyses of the frass in comparison with the pristine Styrofoam to determine whether the degradation of ingested Styrofoam occurred after passage through the superworms' guts. (a) GPC chromatograms of the extract of frass and Styrofoam. (b) ^{13}C CP/MAS NMR spectra of the frass and Styrofoam. The green dash lines indicate the resonance signals (δ 146, δ 128, δ 46 and δ 41) assigned to the PS aromatic carbons or aliphatic carbons. The gray column indicates the resonance signal of newly appearing phenyl derivatives in the frass. (c) TG/DTG curves of the frass and Styrofoam. (d) Three-dimensional infrared (IR) spectra of gaseous compounds evolved from the thermal decompositions of the frass and Styrofoam in the TG equipment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

eat Styrofoam and survives for two months more until they became pupae. Then, the pupae emerged into adult beetles within one week. These results imply that the consumption of Styrofoam diet did not impair, but supported the superworms' activities.

3.2. Degradation of ingested Styrofoam

To determine whether superworms were capable of degrading the ingested Styrofoam in the guts, the fresh frass of Styrofoam-eating superworms were collected and characterized in comparison with the pristine Styrofoam.

Firstly, the THF-extract of frass and the THF-dissolved pristine Styrofoam were analyzed using GPC, respectively. In the GPC profiles (Fig. 2a), the "Fraction a" of frass, representing the long-chain polymeric fraction, appeared behind the "Fraction A" of Styrofoam. Additionally, a new "Fraction b", corresponding to low molecular weight products, occurred later in the extract of frass but not in Styrofoam. The number-average molecular weight (M_n) and weight-average molecular weight (M_w) for the extract of frass also declined compared to that of the Styrofoam (M_n: 66,000 versus 147,000, and M_w: 134,000 versus 274,000). These results suggest that the depolymerization of long chains of PS molecules takes place and the lower molecular weight degradation products are generated in the guts of superworms, which agree with our previous study about biodegradation of PS by mealworms (Yang et al., 2015a).

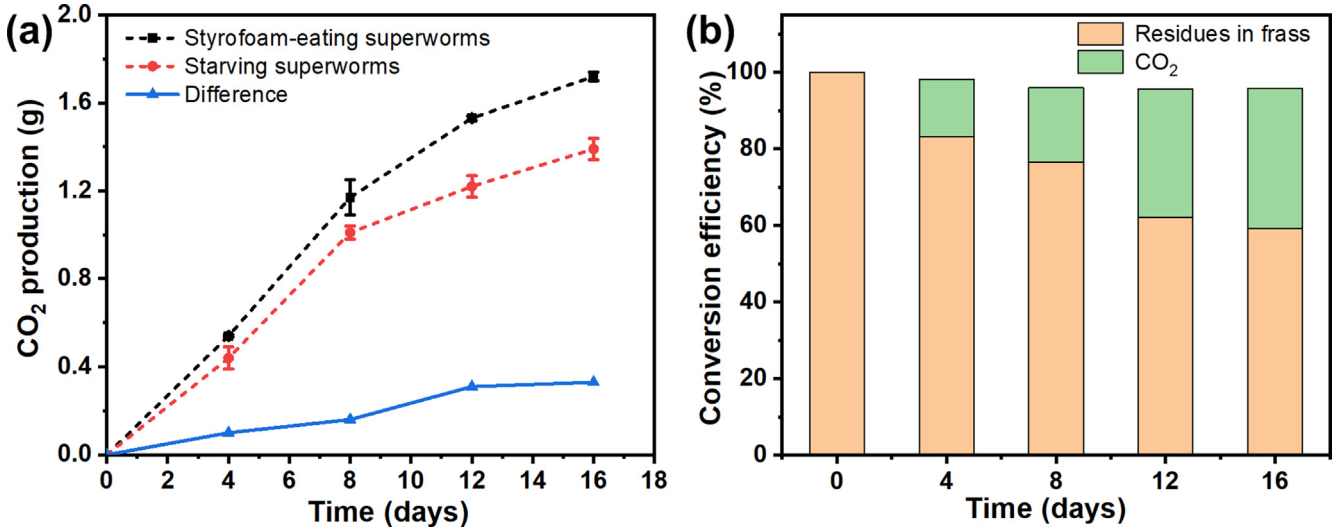

Fig. 3. Conversion of ingested Styrofoam into CO_2. (a) Time course of CO_2 generated from the Styrofoam-eating superworms and starving superworms [mean ± standard deviation (SD); n = 3 groups, 30 worms as a group]. (b) Carbon proportion of the ingested Styrofoam recovered as CO_2 and frass residues based on the carbon balance estimates over a 14-day (mean value; n = 3 groups for each condition, 30 worms for each group).

The chemical compositions of frass and Styrofoam were further characterized using solid-state ^{13}C CP/MAS NMR, which was usually used to directly identify the native chemical composition of substrate without extraction or fractionation of components (Ke et al., 2011). In the spectrum of Styrofoam (Fig. 2b), two strong resonance signals at δ 146 and δ 128 were assigned to non-protonated and protonated aromatic carbons, while another two strong resonance signals at δ 46 and δ 41 were corresponding to the methyl and methylene (aliphatic) carbons. By contrast, the spectrum of frass (Fig. 2b) showed weaker intensities for the four resonance signals (δ 146, δ 128, δ 46 and δ 41) assigned to the PS carbons, indicating a decline in the Styrofoam content in the frass. Additionally, several new resonance signals were detected in the spectrum of frass (Fig. 2b). The resonance signals at δ 160, 154 and 140 could be attributed to aromatic carbons of phenyl derivatives, which maybe the indicated low molecular weight degradation products generated during the degradation of PS. The resonance signals at δ 33 and δ 30 could be assigned to the alkyl- and methyl carbons of hydrocarbons, and the resonance signals at δ 175, 104, 99, 84, 75, 55 and 23 could be referred to carbons in the chitin of the insect cuticle.

The chemical compositions of frass and Styrofoam were also analyzed by the TG interfaced with the online FTIR spectroscopy (TG-FTIR). When the sample was undergoing thermal decomposition during programmed temperature, the weight loss was recorded and the chemical composition of evolved gaseous compounds were online analyzed using FTIR. As shown in Fig. 2c and d, TG/DTG curve of Styrofoam showed only one significant weight loss of 98.0% at 360–480 °C, while the corresponding three-dimensional (3D) FTIR profiles of evolved gaseous compounds at 360–480 °C could be assigned to styrene – the main thermal decomposition product of PS. In comparison, TG/DTG curve of frass showed a less weight loss of 35.4% at 360–480 °C (Fig. 2c), while the absorptions peaks assigned to styrene at 360–480 °C in the 3D FTIR-profile of frass became weaker (Fig. 2d).

Additionally, TG/DTG curve of frass has another two weight loss stages at relative low temperature: stage 1 of 14.4% at 150–250 °C and stage 2 of 19.6% 250–360 °C (Fig. 2c). The absorption peaks at 150–250 °C and 250–360 °C (Fig. 2d) could be assigned to carbon monoxide (1600–1900 cm^{-1}) and carbon dioxide (2200–2400 cm^{-1}), which may evolve from thermal decomposition of the lower molecular weight degradation products of PS (Yang et al., 2015a). These results further demonstrate that the PS component could be significantly degraded and the lower molecular weight degradation products generated after passage through the guts of superworms.

3.3. Mineralization of ingested Styrofoam

To test if superworms could thoroughly mineralize the ingested Styrofoam into CO_2, the CO_2 production associated with the mineralization of Styrofoam was determined by subtracting the CO_2 production of starving superworms (endogenous respiration) from that of Styrofoam-eating superworms. As shown in Fig. 3a, the CO_2 production of Styrofoam-eating superworms was significantly higher than that of the starving superworms over 16 days (30 worms as a group, n = 3 groups; t test, $P < 0.01$). The difference between the CO_2 production of Styrofoam-eating superworms and the CO_2 production of the starving superworms increased as the time of incubation elapsed.

The conversion efficiencies of the ingested Styrofoam to CO_2 and frass residues were estimated by a series of carbon mass balance batch experiments over a number of incubation periods for 4, 8, 12 and 16 days (Fig. 3b). The results showed that total carbon recovery efficiencies were greater than 95%. The carbon of the ingested Styrofoam recovered as CO_2 was increased from approximately 15.1% to 36.7%, while the carbon of the ingested Styrofoam egested as frass was decreased from 83.2% to 59.2% from day 4 to day 16. These results indicate that the mineralization of ingested Styrofoam occurred in the Styrofoam-eating superworms.

3.4. Gut microbiota contributes to Styrofoam degradation

Our previous studies indicated that gut microbiota plays an essential role in the biodegradation of plastic within the mealworms and waxworms (Yang et al., 2015a,b, 2014). As shown in Fig. 4a, a diversity of bacterial cells with various morphotypes (cocci and rods) inhabited within the gut of Styrofoam-eating superworms. To determine if the gut microbiota of superworms also plays a role in PS degradation, we performed an antibiotic suppression assay.

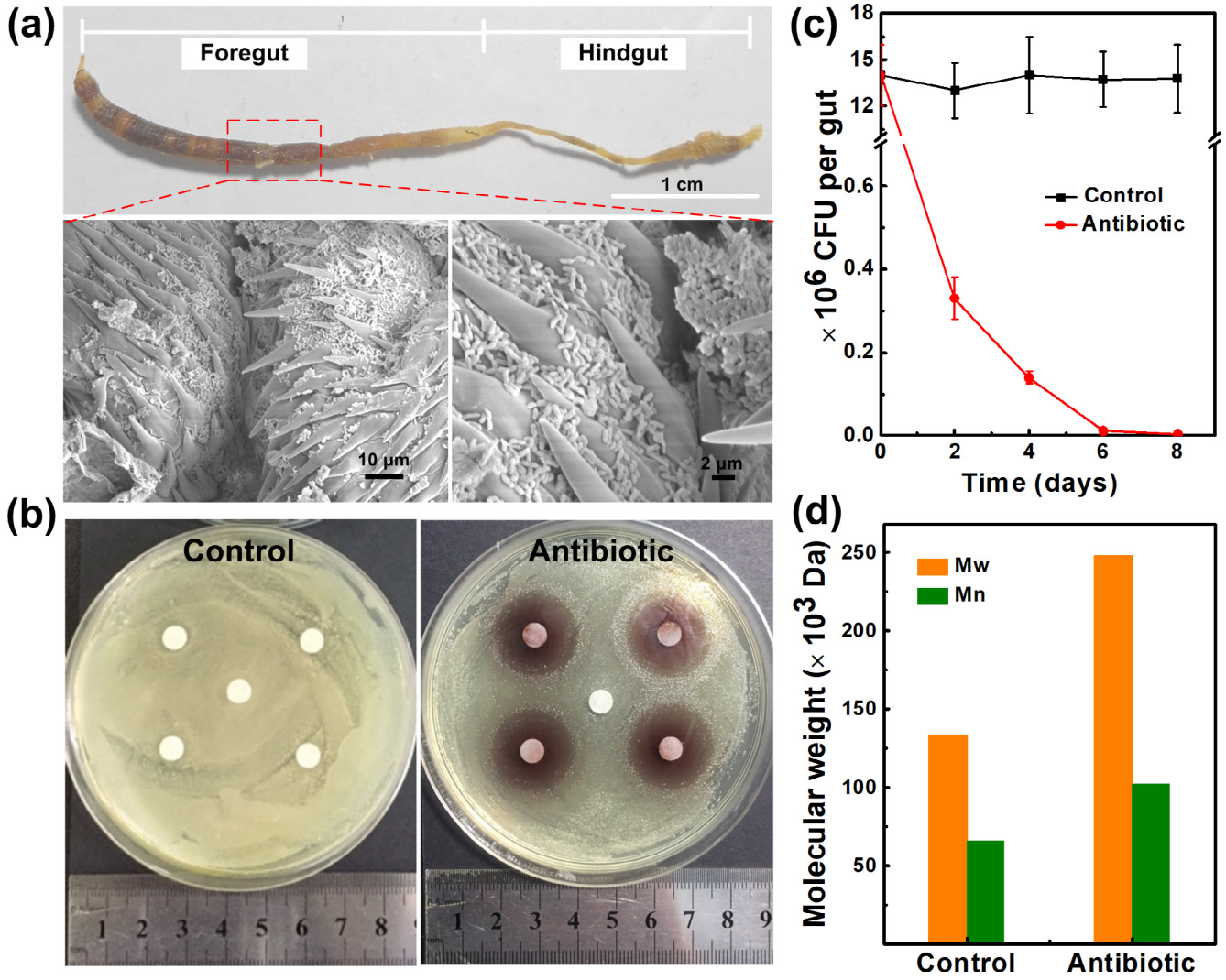

Fig. 4. Contribution of gut microbiota to Styrofoam degradation. (a) Optical photographs of gut structure and ESEM images of gut microbiota that dwelled on the gut wall of superworms. (b) Inhibition halos of the growth of gut microbiota from superworms by the antibiotics (1.6 mg antibiotic per disk, weight ratio, gentamicin:rifampicin: streptomycin = 3:2:6). The disks of control plate and the central disk in antibiotic plate were inoculated with sterile water and didn't form inhibition halo. (c) Number of total visible cells of gut microbiota extracted from superworms fed with antibiotic over an 8-day incubation period. 60 mg of antibiotics (weight ratio, gentamicin:rifampicin: streptomycin = 3:2:6) were mixed with 1 g of bran as antibiotic food, whereas antibiotic-free bran served as the control food. (d) Weight-average molecular weight (M_w) and number-average molecular weight (M_n) of the extract of frass from the Styrofoam-eating superworms untreated or treated with antibiotics.

As can been seen in Fig. 4b, the combination of gentamicin, rifampicin and streptomycin showed a strong ability to inhibit the growth of gut microbiota with the formation of clear halos. Herein, we used the combination of three antibiotics for the suppression of superworms' gut microbiota. When superworms were fed with antibiotic-containing bran (60 mg/g of bran; weight ratio, gentamicin:rifampicin:streptomycin = 3:2:6), the number of active gut microbiota were remarkably suppressed after 8 days (Fig. 4c).

Subsequently, these antibiotic-treated superworms were tested for their PS-degrading capability in comparison with the untreated controls. Both of antibiotic-treated and control (untreated) superworms were fed with Styrofoam, and the average molecular weights of their frass were analyzed. The average molecular weight of the THF-extract of frass egested by antibiotic-treated superworms (M_n = 101,500, and M_w = 248,100) was significantly larger than that of control superworms (M_n = 66,000, and M_w = 134,000) (Fig. 4d). This result indicates that the suppression of gut microbiota by antibiotics impairs the ability of the mealworms to depolymerize PS, and suggests that the gut microbiota plays a potential role in the PS degradation within superworms.

4. Conclusions

Superworms ate Styrofoam as sole diet at the rate of 0.58 mg/d per superworm, which was 4 times more than that of mealworms. After passage through their guts, the ingested long-chain PS molecules were depolymerized into low molecular-weight degraded products, which were further mineralized into CO_2. This new finding confirmed that the plastic-degrading insects extend beyond one specific species and were prevalent in the nature ecosystems. The same physical feature between the mealworms and superworms is the mandibulate mouthpart that enable these species to chew and eat plastic. This feature would inspire us to find more other novel insects capable of chewing and eating plastic.

Eliminating the gut microbial microbiota with antibiotics impaired the superworms' capability of degrading PS. Further studies should focus on the functional diversity of the gut microbiota of superworms. Through metagenomics sequencing-based functional annotations and high-throughput functional screening for enzymatic activity, a variety of novel enzymes for PS degradation may be identified from the plastic-eating insects' gut

microbiota, which could be used as biocatalyst for plastic wastes biodegradation and recycling.

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.2019.135233.

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