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Ingestion preference and efficiencies of different polymerization types foam plastics by *Tenebrio molitor* larvae, associated with changes of both core gut bacterial and fungal microbiomes

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ABSTRACT

The discovery that insect larvae can feed on kinds of foam plastics opened new avenues and provided a potential solution for plastic wastes biodegradation. This study aimed to investigate whether plastic polymerization types had regular impacts on larvae growth, gut microbial communities and functional microbes, foam plastics of polypropylene (PP), polyurethane (PU) and ethylene vinyl acetate (EVA) were selected as the representatives of different polymerized plastic and sole diets for yellow mealworms of *Tenebrio molitor* larvae for 45 days, with sole bran diet as control. Our findings showed that although slightly weight gains were obtained in plastic-fed groups, the larval survival rates decreased to 61.33 %, 59.67 % and 24.00 % in PP-, PU- and EVA-fed groups, respectively. The EVA-fed group was even lower than the starvation group, indicating that the more complex polymers diet had worse adverse effects on larval survival. The gut bacterial- and fungal-microbiomes assessed by Illumina MiSeq indicated that both gut bacterial and fungal communities shifted upon diets of different polymerization types compared to the control. The gut dominant abundances of *Spiroplasma*, *Acinetobacter* and *Pseudomonas* in PP-fed group were significantly different from that of unclassified Enterobacteriaceae in both PU- and EVA-fed groups. In contrast, all gut fungal communities in plastics-fed groups were similar with the dominants of *Rhodotorula* and *Cryptococcus*, but more abundances that had been reported with plastics degradation ability were obtained, such as *Aspergillus* and *Cladosporium*. In summary, *T. molitor* could efficiently degrade complex polymers, albeit with adverse effects. Core gut microbiomes were strongly associated with polymerization types of plastic diet, especially gut fungi.

1. Introduction

Although the damaging effects of insects on plastic packaging were reported in publications as early as 1954 [5], neither plastic waste pollution problem nor plastic biodegradation studies must be paid close attention to at that time. Until 2014, when Dr. Yang first reported that wax insects could feed on and degrade low density polyethylene (LDPE) foam plastics and isolate two PE-degrading bacterial strains from the larval gut [20], resulting in the research on plastics ingestion and degradation by insect larvae the hotspot in the field of plastic biodegradation. Although the biodegradation of LDPE by *Plodia interpunctella* larvae was not verified, the subsequent study firstly confirmed the polystyrene (PS) plastic degradation by *Tenebrio molitor* larvae [25,26].

Currently, many insect worm species have been employed to investigate the ingestion and biodegradation kinds of plastics, such as greater waxworms of *Galleria mellonella* [1,30], superworms of *Zophobas atratus* [9,12,18,17,15], yellow mealworms of *Tenebrio molitor* [11,26,3,6], dark mealworms of *Tenebrio obscurus* [11], and Indian-meal moths of *Plodia interpunctella* [20]. These studies reported the degradation of PP, PU, PS and other polymers by insects. Although the increasing experimental evidences confirmed that the ingested foam plastics were biodegraded by species of insect worms, experiments on multiple types of plastics ingestion by insect worms under the same conditions were rarely reported. Furthermore, such investigations were expected to provide crucial insights into the impacts of plastic polymerization types on insect larval growth, gut microbial communities, and the biological mechanisms underlying plastics degradation in the gut of insect worms.

In this study, we hypothesized that feeding on foam plastics of different polymeric types would differently influence the growth of insect worms and both of the gut bacterial and fungal diversities. To test

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this hypothesis, yellow mealworms were fed with sole foam plastics diets of polypropylene (PP), polyurethane (PU), or ethylene-vinyl acetate (EVA) as representatives of polyolefin, polystyrene and copolymer. The analysis using attenuated total reflection Fourier transform infrared (ATR-FTIR), differential scanning calorimeter and thermogravimetric (DSC-TGA), and gel permeation chromatography (GPC) were employed to confirm the plastic depolymerization. The Illumina MiSeq of both 16S rRNA and ITS genes were employed to uncover the impacts on the changes in gut bacterial and fungal microbial diversities.

2. Materials and methods

2.1. Larvae and feedstocks

Yellow mealworms larvae used in this study were approximately 1.48 ± 0.12 cm in length and 58.28 ± 2.34 mg/larva in weight. Control groups were fed with natural wheat bran without any additives as feedstock. Foam plastics of polypropylene (PP, 0.008 g/cm^3), polyurethane (PU, 0.012 g/cm^3) and ethylene-vinyl acetate copolymer (EVA, 0.021 g/cm^3) as representatives of polyolefin, polyester and copolymer were purchased from specialty stores and used as experimental feedstocks, no plastic was replaced during the experiment. All larvae were fed with bran and starved for 72 h before initiation of the experiment.

2.2. Larval growth and plastics consumption

Four experimental groups were set up with three replicates, including three groups of PP-, PU- and EVA-fed yellow mealworms, with an additional bran-fed control group. For each group, 100 larvae were randomly selected and incubated in a hard texture polypropylene container to maintain a convenient density for good larval growth [14] for 45 d in a controlled dark environment with 25 ± 1 °C and 60 ± 5 % humidity [28,9]. PP, PU, and EVA foam plastics with dimensions of 8 × 5 × 2 cm were cleaned with alcohol and distilled water, and dried at 30 °C for 2 d before the experiment. All larvae were fed with wheat bran for 5 d and then starved for 72 h before switching to the experimental diets. In the control groups, 5 g of bran was added and supplemented every 5 days. One piece of test foam plastic was added to each plastic diet group as sole feedstock for 45 days. Dead larvae and molting exoskeleton were removed immediately via daily checking. Weights of residual plastics and larvae were recorded every 5 days. Larval survival rates, weight changes, and cumulative foam consumptions were calculated at 5-day intervals by comparing to the initial amounts or weights.

2.3. Evidence for foam plastics biodegradation

For confirming foam plastics biodegradation and depolymerization after passing through the larval guts, the analyses using ATR-FTIR, DSC-TGA and GPC were employed [17,19,23,24]. The frass collection and analyses of the residual polymers were performed as described previously [9] in Supplementary (Method S1-S4).

2.4. Gut core microbiome analysis

At the end of the 45-day experiment, 30 yellow mealworms were randomly selected from each group to undergo gut microbiome analysis. Gut microbial genomic DNA was extracted for PCR amplification of the V3-V4 region of the 16S rRNA gene and ITS gene, respectively. The core gut microbial analysis operations were outlined in the Supporting Information (Method S5).

2.5. Statistical analysis

Statistical analyses of larvae survival rates and consumption rates were performed in Prism (version 8) [2]. The pairwise comparisons were performed with the Student's t-test [21]. All *p*-values were adjusted *p*-values and all error values were average ± standard deviation. Statistical significance was set at a *p*-value < 0.05.

3. Results and discussions

3.1. Effects of sole plastic diets on larval weight changes and plastics consumption

Survival rates, weight changes and plastics consumption were used to access the influences of sole plastic diets on the larval growth and development (Fig. 1). The survival rates of each group during the 45-days were calculated (Fig. 1a). At the end of the experiment, the bran-fed control group thread the highest survival rate of 90.67 ± 3.21 %. Whereas both the PP- and PU-fed groups had intermediates of 61.33 ± 2.52 % and 59.67 ± 4.73 %, respectively. Notably, the EVA-fed group demonstrated the lowest of 24.00 ± 5.0 %, which was even lower than the starvation group of 42.0 ± 3.0 %. The significantly lower survival rate in the EVA-fed group was inferred by the inhibition or toxicity of EVA feedstocks. The results highlighted that feeding on sole plastic diet did not fulfill the weight change requirement of yellow mealworms, but the survival rate of the PP and PU group were higher than the starvation group, which also indicated that sole plastic diet could provide some limited source of nutrition for yellow mealworms. Conversely, feeding on EVA had adverse effects on the survival of yellow mealworms, indicating that EVA was not a favorable diet for this species, which was presumed to be due to the toxic effects of EVA plastics.

The weight changes in each group were shown in Fig. 1b. In bran-fed groups, the average weight continuously increased by 108.96 ± 4.41 mg/larva throughout the experiment. The weight changes of plastic-fed groups showed increases during the test with net weight gains of 33.30 ± 1.25, 31.42 ± 2.88 and 28.71 ± 1.08 mg/larva in PP-,

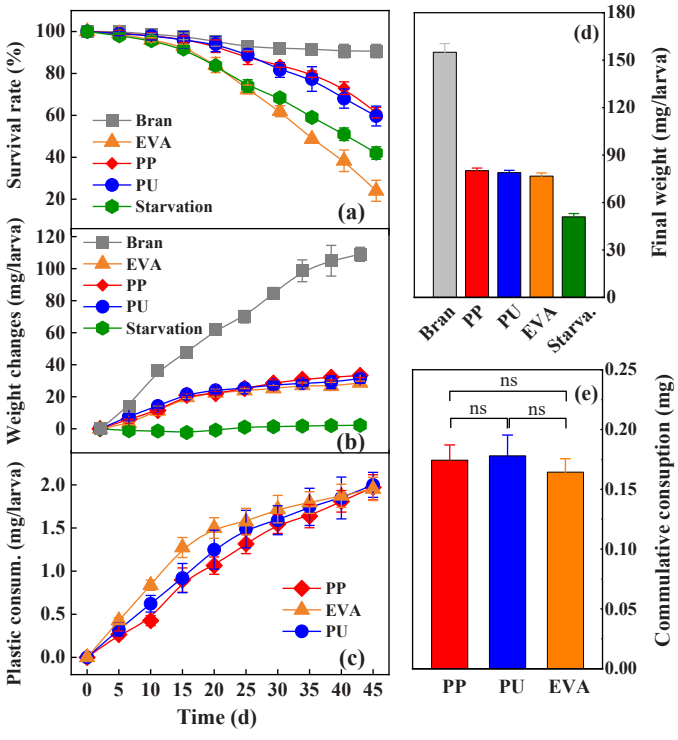

Fig. 1. Different polymerization types foam plastics ingestion by yellow mealworms and effects on larval growth during the 45-day experiment. (a) Survival rates of larvae with different experimental diets; (b) Average net weight changes per larva; (c) Plastics consumption per larva; (d) Accumulative weight changes of yellow mealworms with different sole diet at the experimental end; (e) Cumulative (Cum.) plastics consumption by yellow mealworms. ns: not significant.

PU- and EVA-fed groups, respectively. The results were probably attributed to the smaller size of yellow mealworms and the limited nutritional conditions provided by foam plastics [12]. In addition, the starvation group was also showed weight gain (2.23 ± 0.28 mg/larva), which is inferred that under conditions of food deprivation, yellow mealworms could not satisfy their nutritional requirements and be forced to cannibalize, although the deaths and molting exoskeleton were removed immediately via daily checking in order to avoid the cannibalization.

As shown in Fig. 1c, the cumulative plastics consumption per larva over a 45-day period was consistent across the three polymer types, with values at 1.97 ± 0.15 mg PP, 2.00 ± 0.15 mg PU and 1.95 ± 0.13 mg EVA. These plastic consumption rates were consistent with both larval weight changes and survival rates. As shown in Fig. 1d, after 45 days test, the final weight gains of three plastics-fed groups were almost the same, albeit significantly lower than the bran-fed group and higher than the starvation group. The results indicated that a sole plastic diet could only provide limited energy for the larval weight change. Previous studies had also reported that plastic consumption provided a source of energy and carbon for the life activities of yellow mealworms, but did not help their weigh changes (Yang et al., 2018a, 2018b). Furthermore, no significant difference in the three polymerization types foam consumption ($p < 0.05$) (Fig. 1e), implying that yellow mealworms had equal preference for each plastic type.

3.2. Degradation and oxidation of plastics

The frass pellets of plastics-fed yellow mealworms were collected pellet by pellet, as shown in Fig. S1. Evidences for oxidation and depolymerization, as well as the thermal stabilities changes of PP, PU and EVA by yellow mealworms were obtained and confirmed with ATR-FTIR, DSC-TGA and GPC analyses.

The ATR-FTIR results were shown in Fig. S2 that the functional group of C═O stretch was found in plastic-fed frass, O−H bend and C−O stretch were found in PU-fed frass, while the disappearance of the CH_2 stretch peak in all diet groups, indicating that the depolymerization of the long chain structure of hydrocarbons occurred. Comparing the ATR-FTIR spectra of plastic feedstocks and frass in all diet groups, we speculated that these changes of functional groups should be related to the plastics degradation, PP and EVA plastics were similar to each other but different from PU, indicating the chemical composition and structure differences among different polymerization types of plastics.

The DSC-TGA results (Fig. S3) confirmed the significant differences in the composition and thermal stability of the plastics and the frass, the maximum decomposition rate and weight loss rate of feeding on different polymerization types of plastics showed significant differences, indicating that the plastic components were degraded after passaging through the larval gut. Further, the TGA results also confirmed that polymers with a high complexity were further degraded by gut microorganisms, while polymers with simple composition were relatively lower. The detailed results and discussions were provided in the Supplementary (Results and Discussions S1 & S2, Fig. S2-S3, Table S1-S3).

The GPC analysis of the original plastics and frass of plastics-fed larvae results indicated the significant changes in Mn, Mw and Mz (Fig. S4). For PP- and EVA-fed groups, all Mn, Mw and Mz of the residual polymers increased, respectively. For PU-fed groups, all Mn, Mw and Mz of the residual polymers decreased. The results confirmed that PU plastic was biodegraded via broad depolymerization, while PP and EVA plastic were biodegraded via limited extent depolymerization in yellow mealworms. Moreover, EVA depolymerized more significantly in the larval guts. The results also indicated the complexity and limitation of biodegradation of different polymer types in *T. molitor* larvae. The detailed results and discussions were provided in the Supplementary (Results and Discussions S3).

In summary, based on results of FTIR, TGA and GPC analyses, it was confirmed that the depolymerization and degradation of three different polymerization types of plastics did occur when passing through the gut of yellow mealworms, but with differences in the extent of depolymerization.

3.3. Response of gut-bacterial microbiome

Estimates of gut bacterial species richness and diversity, including OTUs, Chao, Shannon, Simpson, ACE, and Coverage, were presented in Table S4. The OTU number of bran-fed control groups was 180, which decreased to 102 in the PP-fed group, while the PU- and EVA-fed groups showed a slight increase to 186 and 188, respectively. Shannon and Simpson indices also indicated that bacterial richness decreased with a sole plastic diet, especially in PP-fed groups. The decrease of OTUs in PP-fed groups could be attributed to the plastic degradation associated abundances increasing. In contrast, the OTUs increase in PU- and EVA-fed groups were suggested due to the maladaptation of gut bacteria to diets of plastics with complex structures, and the complex structural plastics biodegradation required a joint work of more relevant functional gut bacteria.

Proteobacteria, Tenericutes and Firmicutes were the dominant phyla at the phylum level. Compared to the bran-fed groups, the larval gut bacteria in PP-, PU- and EVA-fed groups significantly shifted ($p < 0.05$) (Fig. S5a), which was mainly associated with the increased relative abundances of Proteobacteria in plastic-fed groups, and the decreased Firmicutes. In the PP-fed group, the community shift was mainly associated with the increased abundances of Tenericute (52.61 %), Fusobacteria (3.10 %) and Bacteroidetes (3.15 %). In the PU-fed group, it was also related to the dominants of Proteobacteria (92.55 %) and Fusobacteria (6.35 %), while, it was related to the Proteobacteria (83.28 %) and Bacteroidetes (8.91 %) in the EVA-fed group. In brief, Proteobacteria was the dominant phyla in PU- and EVA-fed groups, and Tenericutes was the dominant in PP-fed groups.

Based on Unweighted Unifrac analysis, the distinguishable clusters associated with different consumed diets was revealed by PCA (Fig. 2a). The gut bacterial communities were different between the bran-fed and plastics-fed groups, suggesting that plastic ingestion resulted in significant changes in the gut bacteria community. The microbiomes in PU- and EVA-fed groups were similar, and in a different cluster than the PP-fed group, indicating the changes in larval gut bacterial community shaped by diets of plastic types. These findings were further corroborated by heatmap and hierarchical cluster analysis of bacteria (Fig S6a).

The groups differed more significantly at the family level than those at the phylum level (Fig. 2b). Enterobacteriaceae, Enterococcaceae and Streptococcaceae were common families in all groups. In PP-fed groups, compared to the bran-fed group, the dominant families of Spiroplasmataceae, Moraxellaceae and Pseudomonadaceae increased from absent to 53.34 %, 13.29 % and 7.68 %, respectively, indicating their close relationship with the PP diet and significant roles in the PP degradation in guts of yellow mealworms. Enterobacteriaceae was also founded significantly increased in the PP-fed yellow mealworms [22], suggesting its potential association with PP degradation. In PU-fed groups, Enterobacteriaceae was the dominant with a relative abundance of 91.25 %, suggesting its strong correlation with PU foam degradation. In EVA-fed groups, community shift was mainly associated with increased relative abundances of Enterobacteriaceae and Porphyromonadaceae, from 61.82 % and 8.78 % in bran-fed group to 81.85 % and 8.87 %, respectively, assuming to the degradation of EVA foam in the larval guts.

Changes in relative abundances of the top 15 genera were analyzed and presented in Fig. 2c. Unclassified Enterobacteriaceae and *Lactococcus*, the common gut bacteria in yellow and black mealworms [8,24], were also common genera in all test groups in this study. *Pediococcus* and *Weissella* disappeared although they were the dominants in bran-fed groups, and the latter was reported as gut-associated bacteria in mealworms [16]. In PP-fed groups, *Spiroplasma*, *Acinetobacter* and *Pseudomonas* were the dominant genera, accounting for 52.61 %, 9.65 % and

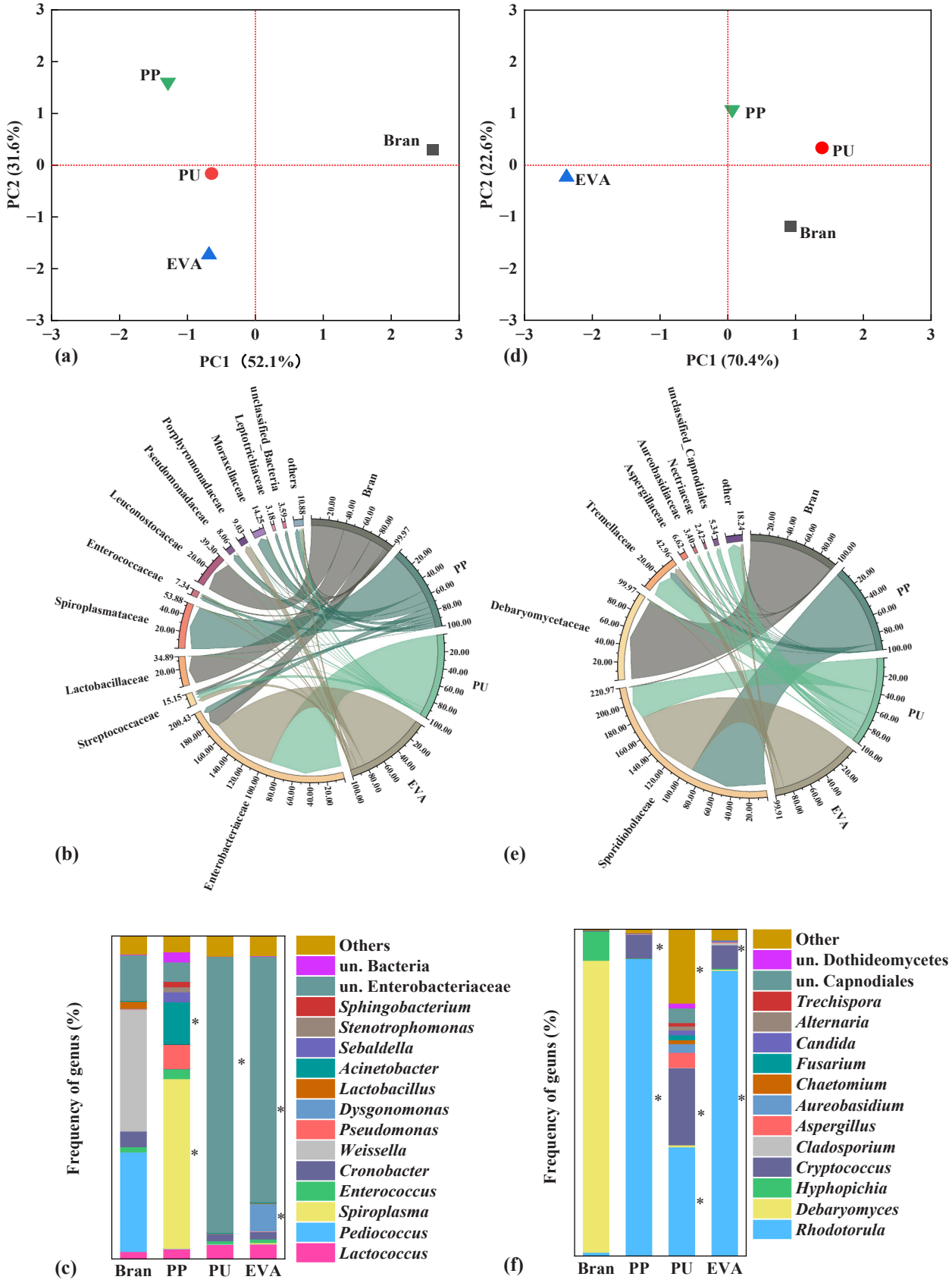

Fig. 2. Analysis of gut microbial diversities of yellow mealworms fed with bran or plastics sole diets. Principal component analysis (PCA) at phylum level of bacterial & fungal (a & d). Relative bacterial and fungal abundances at family level (b & e), and genus level (c & f); * : genera strongly associated with plastic degradation.

7.31 %, respectively, and were suggested to be associated with PP degradation. Among them, *Spiroplasma*, the dominant in PP-fed groups, was significantly higher than that in bran- (<0.01 %), PU- (0.09 %) and EVA-fed (0.40 %) groups, indicating a strong association with the PP biodegradation. Previous studies also reported that it was a common insect gut-associated genus and a known member of the yellow mealworm gut microbiome [2,8]. In PU-fed groups, unclassified Enterobacteriaceae (85.42 %) was observed as the dominant, indicating a strong association with PU degradation. It was also found strongly associated with the degradation of LDPE, PS and PU in superworms of *Z. atratus* [9,18,17,15]. In EVA-fed groups, *Dysgonomonas* and unclassified Enterobacteriaceae were the dominant genera, accounting for 76.11 % and 8.52 %, respectively, which increased by 62.13 % and 8.52 % compared to the bran-fed group. In addition, *Dysgonomonas* was a specific genus associated with the EVA diet, indicating the specific role in the EVA degradation.

In general, compared to the bran diet, sole plastic diet significantly changed the gut bacterial communities of yellow mealworms. However, these changes varied depending on the type of plastic polymerization. The gut bacterial community in polyolefin fed group was significantly differed from those of polyester and copolymer fed group, with *Spiroplasma*, *Acinetobacter* and *Pseudomonas* as the dominant genera. The different bacterial communities of PU- and EVA-fed groups were inferred to be caused by the more complex polymerization structures of polyester and copolymer.

3.4. Response of gut-fungal microbiome

Larval gut-fungi species richness and diversity estimators of OTUs, Chao, Shannon, Simpson, ACE and Coverage were summarized in Table S2. Compared to that in bran-fed groups (19), the OTU numbers of PP-, PU- and EVA-fed groups increased significantly to 52, 123 and 57, respectively. The gut fungal community diversities significantly increased with plastics diets of different polymerization types, especially in PU-fed groups, which was different from that of gut bacteria. Shannon and Chao indices also indicated that fungal richness increased with sole plastic diets, but showed an opposite trend compared to gut bacterial communities.

Three predominant fungal phyla were obtained as shown in Fig. S5b, including Ascomycota, Basidiomycota and Mortierellomycota. Among them, Ascomycota and Basidiomycota were reported to be the two most representatives of fungal phyla that involved in the degradation of various polymers [4]. The relative abundance of Basidiomycota increased in all plastics-fed groups, suggesting a strong association with plastics diets in yellow mealworms. As shown in Fig. 2d, the PCA analysis result indicated that the gut fungal communities were different among EVA groups and other plastic-fed groups. The PP- and PU-fed groups were obtained with similar gut fungal microbiomes, which was also obtained in heatmap and hierarchical cluster analysis (Fig S6b). These results indicated that different polymerization types of plastics caused changes in fungal communities, and the fungal community richness in the polyester plastic-fed group was more apparent.

As shown in the string chart at the family level (Fig. 2e), Debaryomycetaceae was the dominant family in bran-fed groups but less than 1 % in plastics-fed groups, while Sporidiobolaceae and Tremellaceae were the dominant families in plastic-fed groups. Interestingly, significant differences in dominant families were also observed among the plastic-fed groups. For example, the Aspergillaceae (7.47 %) was the dominant in PP-fed groups, Aspergillaceae (6.03 %) and Aureobasidiaceae (3.11 %) were the dominants in PU-fed groups. In EVA-fed groups, the relative abundance of Sporobacteriaceae was 88.55 %, which increased by 87.44 % compared to the bran-fed group.

Changes of the top 14 fungal genera relative abundances with > 1 % were analyzed (Fig. 2f). Two OTUs of *Hyphopichia* and *Debaryomyces* were the dominants in the bran-fed group, accounting for 89.34 % and 8.95 %, respectively, but these two fungal strains probably with high carbohydrate metabolism efficiency decreased or disappeared in all plastics-fed groups. Relative abundances of *Rhodotorula* and *Cryptococcus* significantly increased, and reached 7.19 % and 87.36 % in PP-fed groups, 7.40 % and 91.05 % in PU-fed groups, 23.57 % and 33.36 % in EVA-fed groups, respectively, in comparison to bran-fed groups of < 0.01 %. *Rhodotorula* and *Cryptococcus* were found to be specific functional groups uniquely associated with plastic degradation, which were strongly associated to both PE and PS degradation in yellow mealworms [18,17,15].

In our study, *Cryptococcus* and *Rhodotorula* were strongly associated with PP, PU & EVA diet, demonstrated their involvement in the biodegradation of plastics. In addition, the microbial abundances of PU diets were higher compared to bran diets, in exception to the common fungal populations, it also included increased abundance of *Aspergillus* and *Aureobasidium*. The possible reason for this result was the unique structure of polyester plastic, which was composed of polyisocyanates and polyols containing C═O bonds and nitrogen. This composition probably leaded to greater enrichment of fungal colonies in the guts of yellow mealworms. Moreover, the degradation of PU was more dependent on the production of extracellular enzymes than other polymeric types of plastics [7,10,13,29]. A higher fungal community richness was observed in the PU-fed group, indicating that the fungi in the gut could secrete more enzymes and contribute more in the slightly higher PU plastic biodegradation. Lastly, the above results also indicated that feeding on different polymeric plastics would cause fungal colony significant changes in gut of yellow mealworms, in which, fungal communities in the polyolefin- and copolymer-fed groups had slight similarities.

Overall, the core gut functional microbiome compositions were related to the plastics diets of polymerization types. Compared to the gut bacteria, feeding on plastics with higher complexity resulted in greater changes in the gut fungal community of yellow mealworms, but with similar predominant abundances. Besides the dominants of *Rhodotorula* and *Cryptococcus* obtained in all plastics-fed groups, more abundances such as *Aspergillus* that had been reported with plastics degradation ability were observed in PU-fed groups, indicating the association of core gut fungi and plastics biodegradation. Sole plastic diets of different polymerization types significantly altered the gut microbial community structure of yellow mealworms, although the trends were distinct. The responses of gut bacteria and fungi to plastics polymeric types were different, and the plastics biodegradation process in gut of insect worms were a joint process achieved with both functional bacteria and fungi.

4. Conclusions

All test foam plastics of PP, PU & EVA, as the representatives of different polymerized plastic types, could be ingested and biodegraded by yellow mealworms, but had obvious adverse effects on the larval weight change and even survive, especially the EVA foam with the most complex structure. Both of gut bacterial and fungal communities of yellow mealworms shifted upon sole diets of different polymerization types compared to the control, albeit in different trends. The responses of gut bacteria and fungi to plastics polymeric types were different, and the plastics biodegradation processes in gut of insect worms were suggested to be joint works achieved with different gut functional bacteria and fungi. Importantly, this study was the first-time report on EVA foam biodegradation by yellow mealworms. Future studies on isolation of both individual bacteria and fungi species, and their cocultures relation to plastic degradation process will be benefit the clarification of the molecular mechanisms involved in plastic biodegradation and the future applications.

CRediT authorship contribution statement

Yijing Wang: Investigation, Writing – original draft. **Xin Zhao:** Supervision, Writing – review & editing, Funding acquisition. **Jiaming**

Wang: Investigation. **Yue Weng:** Methodology. **Yumeng Wang:** Investigation. **Xin Li:** Investigation. **Xiaoyu Han:** Writing – review & 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.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2023.110801.

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