

Biodegradation of Expanded and Extruded Polystyrene with Different Diets by Using *Zophobas atratus* Larvae (Coleoptera: Tenebrionidae)

Jun Hoe Tay¹, Norhayu Asib², Nor Azwady Abd Aziz³ and Geok Hun Tan^{1*}

¹Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

²Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

³Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Polystyrene waste pollutes the environment and poses a significant health risk to humans, animals, and marine ecology. This study aims to evaluate the effectiveness of degradation on expanded (EPS) and extruded (XPS) polystyrene with different diets using superworms (*Zophobas atratus* larvae) obtained in Malaysia. The growth and development of the larvae after consumption of EPS and XPS and the gut microbial community changes in response to high polystyrene consumption diets were also identified. The oatmeal, wheat bran, and cornmeal were used as supplement diets and showed significantly enhanced EPS and XPS consumption and degradation compared to sole diet treatment. Gel permeation chromatography was carried out using egested frass of *Z. atratus* larvae to characterize depolymerization of EPS and XPS, indicating a significant reduction in the average molecular weight and average molecular weight. The highest reduction occurred in the presence of oatmeal. Proton nuclear magnetic resonance and Fourier transform infrared spectroscopy analyses indicated functional group changes and chemical modification occurred with

depolymerization and partial oxidation of EPS and XPS. The larvae length increased, while the number of instars and duration of larvae became shorter with the addition of supplement diets. Oatmeal is predominantly effective among other supplements in assisting *Z. atratus* larvae with EPS and XPS degradation. The results of this study support the ubiquity of polystyrene biodegradation in *Z. atratus* and the next-

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E-mail addresses:

junhoe91@hotmail.com (Jun Hoe Tay)

norhayuasib@upm.edu.my (Norhayu Asib)

azwady@upm.edu.my (Nor Azwady Abd Aziz)

geok_hun@upm.edu.my (Geok Hun Tan)

* Corresponding author

generation sequencing studies. *Kluyvera* sp., *Klebsiella* sp., and *Enterobacter* sp. were found to be strongly associated with degrading EPS and XPS polystyrene with oatmeal as a supplemental diet.

Keywords: Biodegradation, expanded polystyrene, extruded polystyrene, superworms, supplements, *Zophobas atratus*

INTRODUCTION

Plastic production worldwide is increasing yearly (Shanmugam et al., 2020). However, plastic waste takes the longest time to decompose naturally among different waste products. Improper plastic waste management, such as polystyrene, has caused accumulation in every corner of the world, adversely affecting wildlife and human health (Khoo et al., 2021). Besides, plastic trash accumulates in the sea creatures (Thushari & Senevirathna, 2020).

Insect larvae belonging to darkling beetles (Tenebrionidae), e.g., yellow mealworms (*Tenebrio molitor*) and superworms (*Zophobas atratus*), were found to degrade various plastic, especially polyethylene (PE), polystyrene (PS), and polypropylene (PP), among others, since 2015 (Brandon et al., 2018; Peng et al., 2019, 2022; Y. Yang et al., 2015, 2020; Yang, Brandon, et al., 2018; Yang, Wu, et al., 2018). The ubiquity of PS biodegradation of *T. molitor* was confirmed via tests of the larvae worldwide (Yang, Wu, et al., 2018). *Zophobas atratus* Fabricius 1775 (Coleoptera: Tenebrionidae) is a superworms commercially available animal feed worldwide (Rumbos & Athanassiou,

2021). Recently, *Z. atratus* strains from China and USA were reported to biodegrade PS (Peng et al., 2020, 2022) and low-density polyethylene (LDPE) via a gut microbial-dependent mechanism (L. Yang et al., 2021). However, the plastic degrading ability of *Z. atratus* in other areas has not yet been elucidated. Meanwhile, the effect of expanded polystyrene (EPS) and extruded polystyrene (XPS) consumption with different feeding diets on *Z. atratus* larvae remains unclear. Oatmeal, wheat bran, and cornmeal were used as a supplemental diet for *Z. atratus* larva to evaluate the consumption of EPS and XPS. Oatmeal, wheat bran, and cornmeal rich in essential nutrients are common feeding diets for *Z. atratus*. Several studies showed that oatmeal, wheat bran, and cornmeal manipulated the guts microbiota of *T. molitor* larvae and enhanced polystyrene consumption (D. Zhou et al., 2021; Gao et al., 2010; Matyja et al., 2020). Yang, Brandon, et al. (2018) first reported enhanced PS consumption by *T. molitor* larvae due to co-feeding bran and protein powders.

Insects asylum diverse microorganisms in the guts benefit their host, physiologically and ecologically (Singh et al., 2019). The major function of microorganisms harboring in the gut is to digest ingested food (Jang & Kikuchi., 2020). The capacity of *Z. atratus* and *T. molitor* larvae to degrade polystyrene (PS) within the gut concerning microorganism activity has been confirmed recently (S.-S. Yang & Wu, 2020; Y. Yang et al., 2015, 2020; Yang, Wu, et al., 2018). Thus, studying the *Z. atratus* larvae gut microbial community on the high

polystyrene (XPS and EPS) consumption diets is worth exploring.

This study investigated the supplementation of different co-diet to enhance *Z. atratus* larvae to degrade EPS and XPS. Besides, the growth and development of *Z. atratus* larvae after consuming expanded (EPS) and extruded (XPS) polystyrene was also evaluated. The gut microbial population alterations in response to high polystyrene consumption diets were also studied.

MATERIALS AND METHODS

Sources of *Zophobas atratus* Larval and Test Materials

Zophobas atratus beetles (120 individuals) with an average of 22.78 ± 2.36 mm length/individual and 500.30 ± 74.00 mg/individual were purchased from Ban Lee Agro (M) Sdn. Bhd. (Rawang), Malaysia then reared into a plastic container (18 cm length \times 12 cm width \times 7 cm height). After *Z. atratus* beetles undergo oviposition, the eggs are collected and allowed to hatch before the experimental study. A newly hatched *Z. atratus* larvae were immediately isolated into each plastic container (18 cm length \times 12 cm width \times 7 cm height) in a controlled environment (temperature: $28 \pm 1^\circ\text{C}$ and relative humidity [RH]: 50–70% RH). All larvae were healthy, without defects, and free from disease, antibiotics, additives, and hormones.

EPS (0.0375 g/cm³ density) and XPS (0.1086 g/cm³ density) polystyrene were used as a feedstock purchased from Yee Hup Foam and Packaging Industries Sdn.

Bhd. (Shah Alam, Malaysia). Throughout gel permeation chromatography (GPC) analysis, the weight average molecular weight (Mw) and number average molecular weight (Mn) values of EPS were 399.90 ± 1.54 and 98.50 ± 0.96 kDa, respectively. Meanwhile, XPS polystyrene has Mw and Mn values of 360.60 ± 0.60 and 83.50 ± 0.67 kDa. Before the test, sterilized distilled water was used to clean the EPS and XPS polystyrene components, then dried for 48 hr at 30°C to ensure cleanliness and dirt removal.

Oatmeal (66.60% carbohydrates, 16.70% protein, 5.80% fat, 10.80% fiber, and others), wheat bran (64.20% carbohydrates, 15.80% protein, 4.20% fat, 12.90% fiber, and others), and cornmeal (76.70% carbohydrates, 8.30% protein, 3.30% fat, 7.50% fiber, and others) with 1.2 g, respectively, were used as supplements in the feedstock according to the treatment. All supplements were purchased from Ban Lee Agro (M) Sdn. Bhd. without adding pesticides and antibiotics. All supplement foods were sorted and appropriately filtered to eliminate impurities and contamination before the experiment.

Biodegradation of Expanded Polystyrene and Extruded Polystyrene

EPS and XPS polystyrene (1.2 g) used for each replicate according to treatment were washed with clean air to eliminate dirt particles. An isolated single *Z. atratus* larva was grown in each treatment container. During the cultivation period, *Z. atratus* larvae were raised in a controlled environment at $28 \pm$

1°C with a relative humidity of 50–70%, according to Yang, Brandon, et al. (2018), with slight modifications. Eight treatments were prepared according to the feeding conditions (Table 1): i) EPS only; ii) EPS + oatmeal; iii) EPS + wheat bran; iv) EPS + cornmeal; v) XPS only; vi) XPS + oatmeal; vii) XPS + wheat bran; and viii) XPS + cornmeal, respectively. Oatmeal, wheat bran, and cornmeal as supplements were supplied only once with 1.2 g, respectively, in the selected treatments. EPS and XPS consumption were identified based on the mass loss via weighing the unconsumed EPS and XPS within treatments. All treatments were carried out with sixty replicated.

Characterization of EPS and XPS Biodegradation with Frass Contents

Gel permeation chromatography with high temperature (HT-GPC) (Agilent 1260 Infinity II GPC/SEC system, USA) was used to identify the Mw and Mn of digested polystyrene in frass contents with minimal

modifications based on Yang, Brandon, et al. (2018). Sample (frass) around 50 mg was deposited into a sterile glass vial (30 ml) that contained 10 ml tetrahydrofuran (THF) (Merck, Germany) to extract for 2 hr at 30°C. The extract was filtered twice using a polyvinylidene fluoride (PVDF) sterile syringe filter with 0.22 µm (Bioflow, Malaysia). THF extract samples (100 µl) were inserted into a GPC running at 40°C with a THF eluent 1.0 ml/min (flow rate). The results of molecular weight (Mn and Mw) were recorded.

Proton nuclear magnetic resonance (¹H NMR) was used to examine the chemical changes between raw polystyrene and frass samples based on the Peng et al. (2020) protocol. Fresh frass samples (50 mg) were deposited in glass vials (10 ml), then 2,000 µl chloroform-D (Merck, Germany) was added and allowed thoroughly mix for 2 hr. The extracts were transferred to a clean glass vial (10 ml) after being filtered with PVDF sterile syringe filter with 0.22 µm (Bioflow

Table 1
The treatment used for the experimental study

Treatment	Information
Group 1 (Expanded polystyrene)	
T1	Expanded polystyrene only
T2	Expanded polystyrene + oatmeal (ratio 1:1)
T3	Expanded polystyrene + wheat bran (ratio 1:1)
T4	Expanded polystyrene + cornmeal (ratio 1:1)
Group 2 (Extruded polystyrene)	
T5	Extruded polystyrene only
T6	Extruded polystyrene + oatmeal (ratio 1:1)
T7	Extruded polystyrene + wheat bran (ratio 1:1)
T8	Extruded polystyrene + cornmeal (ratio 1:1)

Note. Expanded polystyrene = 1.2 g; Extruded polystyrene = 1.2 g; Supplements (oatmeal, wheat bran, cornmeal) = 1.2 g. The experiment was recorded until pupation took place

Lifescience, Malaysia). Extracts (600–700 μl) were injected into the 1,000 μl NMR tubes. The ^1H spectra were measured using a Varian Inova 500 MHz NMR spectrometer (USA). The ^1H spectra were expressed in ppm, also known as parts per million, with a reference peak for residual chloroform-D (^1H –7.26 ppm).

The alterations in the major functional groups of polystyrene were identified using Fourier transform infrared spectroscopy (FTIR), known as Fourier Transform Infrared Spectroscopy (Brandon et al., 2018; Sekhar et al., 2016). FTIR spectroscopy (Perkin Elmer, Spectrum 100, USA) examined the frass samples. The absorbance was measured in the range of 650–4,000 per cm wavenumbers in the mid-IR region. A comparison among treatments was recorded when different peaks occurred, such as C=O carbonyl group, =C-H bend, C-O stretch, methylene (CH_2) deformation, C=C stretch (benzene ring), CH_2 bending (symmetrical), and CH_2 bending (asymmetrical).

Growth and Development of *Z. atratus* after Ingested Polystyrene

The growth and development of larvae after ingested polystyrene was investigated based on measuring body length (mm). The larvae length (mm) was measured using a digital vernier caliper (Senator DCS 200, Germany) after larval exuvium was observed (Kim et al., 2015). The larva length was measured from the tip of the head thorax until the end of the abdomen. During larva measurement, avoid direct contact because *Z. atratus*

larvae are susceptible to external stimuli. It will shrink when touched, resulting in measuring errors.

The number of instars and total duration of larval instars (days) were recorded after the larval exuvium was observed. The exuvium of larvae was discarded after being observed to avoid duplicate counting. The number of instars and larval development duration can vary depending on food quality (Morales-Ramos et al., 2010). After pupation, the larvae-initiated curling and the number and duration of larval instar counting were stopped.

Microbial Community Analysis

The treatment focuses on potential supplement diets that effectively consume EPS or XPS. Three treatments were prepared according to the feeding conditions: (1) oatmeal only, (2) EPS + oatmeal, and (3) XPS + oatmeal, respectively. Twenty larvae with 10 to 12 instars from each treatment were randomly harvested to avoid individual variability between larvae with identical diets. The experiment was repeated three times. Larvae were collected to make a gut cell suspension used as a bacterial enrichment inoculum. The surfaces of the larvae were immersed in 70% absolute ethanol (Merck, Germany) for 60 s to eliminate surface germs. The larvae were washed with sterilized distilled water (Hu et al., 2018). The protocol by Brandon et al. (2018) and J. D. Zhou et al. (1996) was modified and used for bacterial genomic DNA extraction. Scalpel and clipper removed the guts walls from the

outer skin layer (cuticle). Guts walls were discarded, and DNA was extracted by mixing guts contents with 500 µl of 2% (w/v) sodium dodecyl sulfate (SDS, Merck, Germany) and lysis solution of 500 µl (ethylenediaminetetraacetic acid [EDTA, 0.5 M]) with pH 8.0, cetyltrimethylammonium bromide (CTAB, 0.1 M), Tris-hydrochloric acid (Tris-HCl, 10 Mm) with pH 7.5, sodium chloride (NaCl, 0.3 M), respectively in a microcentrifuge tube (2.0 ml) then vortexing for uniformly mixed.

The suspension formed was incubated at 37°C for 20 min in the water bath. The suspension was mixed with the same volume of phenol (Merck, Germany), chloroform (Merck, Germany), and isoamyl alcohol (Merck, Germany) with the ratio of 25:24:1 into a new 2.0 ml microcentrifuge tube and then centrifuged for 10 min with 12,298 x g (Eppendorf, Centrifuge 5415 R, Germany). The liquid formed at the aqueous phase was aliquoted into a new microcentrifuges tube (2.0 ml). The same amount of chloroform and isoamyl alcohol (24:1) was added and mixed well, then centrifuged for 10 min at 12,298 x g. The aqueous phase (top layer) containing genomic DNA was precipitated by adding 100% cold absolute ethanol (2.5 volumes) and mixed well. The mixture containing genomic DNA was incubated overnight at -4°C in the refrigerator. After overnight precipitation, the mixture was centrifuged for 10 min at 12,298 x g. The supernatant was discarded while the pellet consisting of genomic DNA was washed with 70% (v/v) cold absolute ethanol (700 µl) (Merck, Germany). The pellet containing 70% (v/v) absolute ethanol was centrifuged

for 10 min at 12,298 x g and then allowed to air-dry in laminar airflow. The dried DNA pellet was then dissolved in 40 µl of TE buffer [Tris-HCl (10 mM) with pH 7.5 and ethylenediaminetetraacetic acid (1 mM with pH 8.0, Merck, Germany)]. The genomic DNA was then kept in a -20°C freezer.

The 16S rRNA gene in the V3-V4 region was sequenced using phasing amplicon sequencing (PCoA), as described by S.-S. Yang et al. (2021), with some modifications. Illumina MiSeq platform (next-generation sequencing [NGS]) was used to paired-end sequenced on purified amplicons. UCHIME (v4.2.4.0) was used to remove low-quality sequences. Ribosomal Database Project (RDP) used a 70% confidence level to evaluate the taxonomic of each 16S rRNA gene sequence against the SILVA 16S rRNA database. R packages (v3.4.3) and QIIME (v1.9.1) were used to run alpha diversity, principal coordinate analysis, and relative abundance analyses according to taxonomic tanks and operational taxonomic unit (OTU) sequences.

Statistical Analysis

All the research data was gathered and processed using statistical analysis (SAS) software and analysis of variance (ANOVA). The mean separation of treatment was done using Tukey's test to access total plastic consumption amount and changes in molecular weight, differences in body length, total number and duration of larval instars, and microbial diversity between diets. The study was conducted using a significance threshold of $p < 0.05$.

RESULTS AND DISCUSSION

EPS and XPS Polystyrene Consumption by *Z. atratus* Larvae

The study of the EPS and XPS groups revealed that differing diets had a significant influence ($p < 0.05$) on polystyrene consumption (Figure 1). Treatments utilizing oatmeal, wheat bran, and cornmeal as co-diet demonstrated a substantial polystyrene consumption by *Z. atratus* larvae in EPS and XPS groups. This phenomenon has proven that including nutrient-dense meals in one's diet may increase the consumption of EPS and XPS. Treatment EPS (oatmeal) as a co-diet produced higher total EPS consumption, 422.30 ± 21.44 mg, compared to treatment EPS only (57.40 ± 6.05 mg). In the XPS group, XPS (oatmeal) as a co-diet showed the highest total plastic consumption, 268.33

± 11.08 mg, compared to treatment XPS only (43.73 ± 5.70 mg). The interaction of nutrients with the microbiome may impact the larva's behavioral decisions (Leitão-Gonçalves et al., 2017). Oatmeal has slightly higher essential nutrient compositions. Most importantly, it may help improve larva appetite and increase the specific variety of microbiota in the *Z. atratus* larva gut to consume EPS and XPS efficiently (Fu et al., 2020). Peña-Pascagaza (2020) and Yang, Brandon, et al. (2018) revealed that EPS and XPS polystyrene contain only hydrogen and carbon elements, thus unfavored polystyrene consumption by larvae. Treatment EPS (wheat bran) showed significantly higher EPS consumption (220.96 ± 10.17 mg) than EPS (cornmeal), with 91.17 ± 4.87 mg within the EPS group.

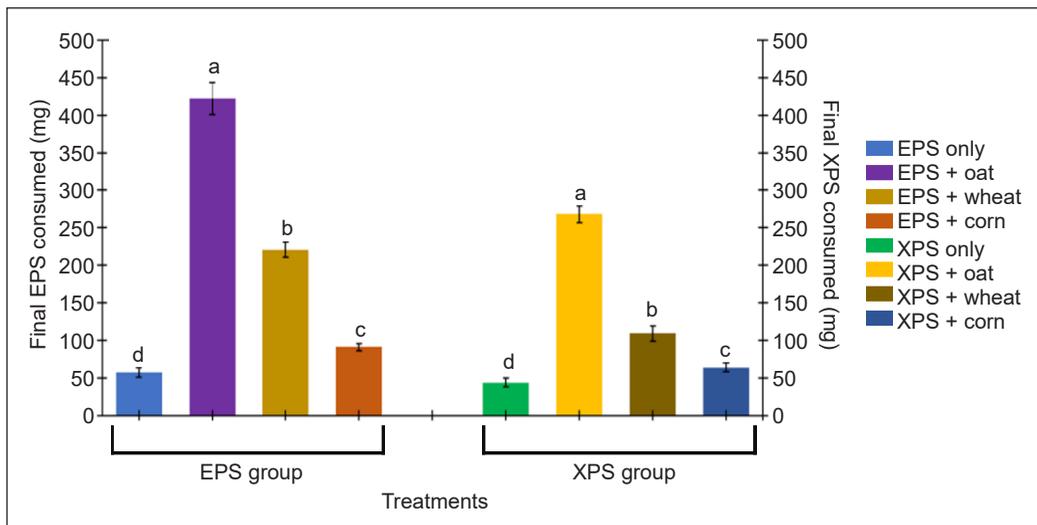


Figure 1. Final plastic consumption of larva in each treatment with EPS group and XPS group. Electronic balance was used to measure the plastic left by larval

Note. Initial EPS / XPS mass = 1.2 g; Supplement (oatmeal/wheat bran/cornmeal) mass = 1.2 g. The experiment was recorded until the pupation took place. All values were obtained from the mean of sixty replications with larvae reared individually and separately per replication. Mean followed by different letters are significantly different at $p < 0.05$ level according to Tukey's one-way ANOVA. The same letter above the bar within the group represents no significant difference between treatments

In the XPS group, the treatment XPS (wheat bran) also showed significantly higher XPS consumption (109.12 ± 10.07 mg) than treatment XPS (cornmeal) with 63.97 ± 5.80 mg. Lou et al. (2020) discovered that wheat bran as a co-diet may modify the gut microbiota of *T. molitor* larvae and improve the capacity to break down polyethylene and polystyrene. Wheat bran has slightly lower essential nutrient compositions than oatmeal, reducing the favoring of microbiota production and larvae appetite on EPS and XPS consumption. Besides, cornmeal easily absorbs moisture from the surroundings, influencing shelf life and nutrient contents (McHargue, 1920). Thus, treatment with cornmeal as a co-diet with insufficient nutrients provided the growth of the gut microbiome, reducing EPS or XPS consumption.

Evidence of EPS and XPS Polystyrene Biodegradation Through Frass Contents Analysis

GPC was carried out using egested frass of *Z. atratus* larvae to characterize the depolymerization of EPS and XPS (Peng et al., 2020). The GPC method determines linear polymers' molecular weight (Mw and Mn), particularly polystyrene (Kissin, 1995). GPC results revealed significant changes ($p < 0.05$) in Mw and Mn of the residual EPS and XPS polymers (Figure 2). Mn and Mw in EPS and XPS groups showed decreased values after EPS, and XPS degraded in the gut of *Z. atratus* larvae in all treatments. In the EPS group, GPC analysis on residual polymer showed that treatment EPS (oatmeal) produced the highest reduction with Mw ($49.08 \pm 2.08\%$) and Mn ($28.24 \pm 0.43\%$) compared among

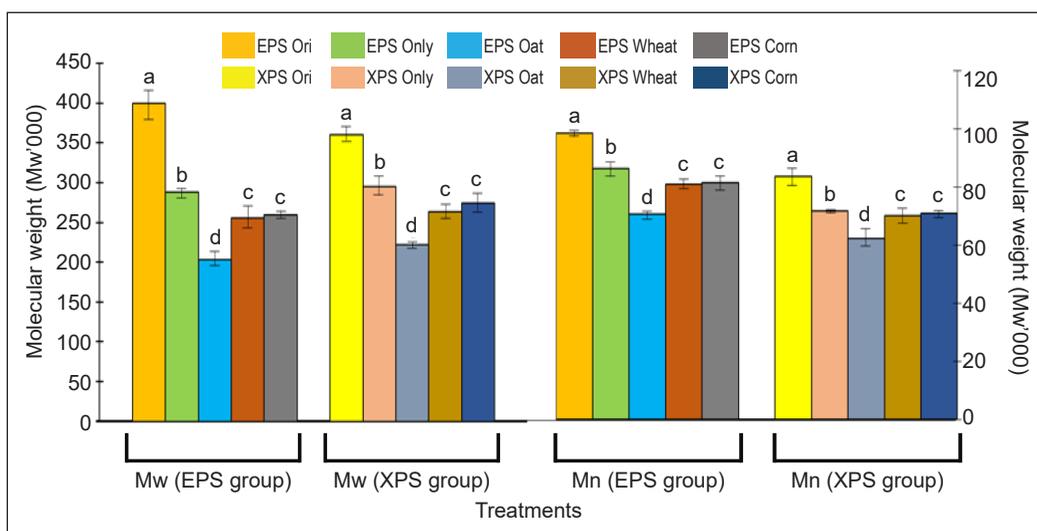


Figure 2. Weight average of molecular weight (Mw) and number average of molecular weight (Mn) of residual polymer from larval frass with treatment EPS group and XPS group
 Note. GPC analytical technique was used to measure the molecular weight of degraded polymer after passing through the larval gut passage. Mean followed by different letters are significantly different at $p < 0.05$ level according to Tukey's one-way ANOVA. The same letter above the bar within the group represents no significant difference between treatments

the EPS group. Meanwhile, the XPS group with treatment XPS (oatmeal) produced the highest reduction with Mw ($38.37 \pm 2.60\%$) and Mn ($25.35 \pm 0.33\%$) compared to the XPS group. Oatmeal with essential nutrients supports larvae growth and enhances EPS and XPS degradation. The higher Mw and Mn value reduction indicated degradation activity and depolymerization were highly effective than other treatments. The enhanced variety of gut microbial activity is responsible for the capacity to depolymerize polystyrene (Fu et al., 2020). Besides, treatment EPS (wheatmeal) and EPS (cornmeal) are insignificant, but both treatments showed significant residual polymer reduction in Mw and Mn when compared with the EPS original. The results obtained were similar to the XPS group as well. XPS (0.1086 g/cm^3) is generally denser than EPS (0.0375 g/cm^3). It could be why more PS mass was consumed and higher depolymerization extent of EPS (27.9% Mw, 12.4% Mn reduction) than XPS (18% Mw, 9.3% Mn reduction), although both foams had similar Mw and Mn. When ingested by larvae, wheat bran contains essential nutrients, triggering the gut microbiome to metabolize polystyrene (Brandon et al., 2018). Cornmeal also plays a similar role in helping *Z. atratus* larva metabolize polystyrene. However, wheat bran and cornmeal can enhance the reduction of Mw and Mn but are less efficient than oatmeal as supplements. The difference in PS consumption and depolymerization extent between oatmeal vs. wheat bran or cornmeal could be the difference in protein

content of the co-diets. The results of GPC analyses indicated that both EPS and XPS degradation in *Z. atratus* larvae tested were performed via a broad depolymerization pattern or decrease in both Mw and Mn (L. Yang et al., 2021; Peng et al., 2022).

A comparison of FTIR spectra showed evidence of chemical changes and new functional group formation, indicating oxidation of residual polymer (Lin & Liu, 2021; Umamaheswari & Murali, 2013). Evidence of polystyrene degradation can be shown by monitoring the reduction in peak intensities and the new peak formation compared to the original polystyrene (J. Yang et al., 2014; Sekhar et al., 2016). The spectrum of residual polymer from *Z. atratus* larvae fed with EPS and XPS treatment showed decreased peak intensities, and a new functional group occurred compared with the original EPS (EPS group) and XPS (XPS group) (Figures 3 and 4). In the EPS group, the peaks with (C=C stretch, 1,551 to 1,610 per cm) are known as benzene rings (Yang, Brandon, et al., 2018). It was found that treatment EPS only, EPS (cornmeal), EPS (wheat bran), and EPS (oatmeal) showed a decrease in intensity accordingly and dampened, indicating the evidence of ring cleavage. Besides, the -C-O stretch and -C=O carbonyl were responsible for the peak at 1,736 per cm and the lesser intensity at about 1,085 per cm. This peak was found in all EPS treatments, and EPS (oatmeal) treatment showed the most significant EPS degradation with new peaks and decreasing intensity compared to different treatments. This peak showed signs

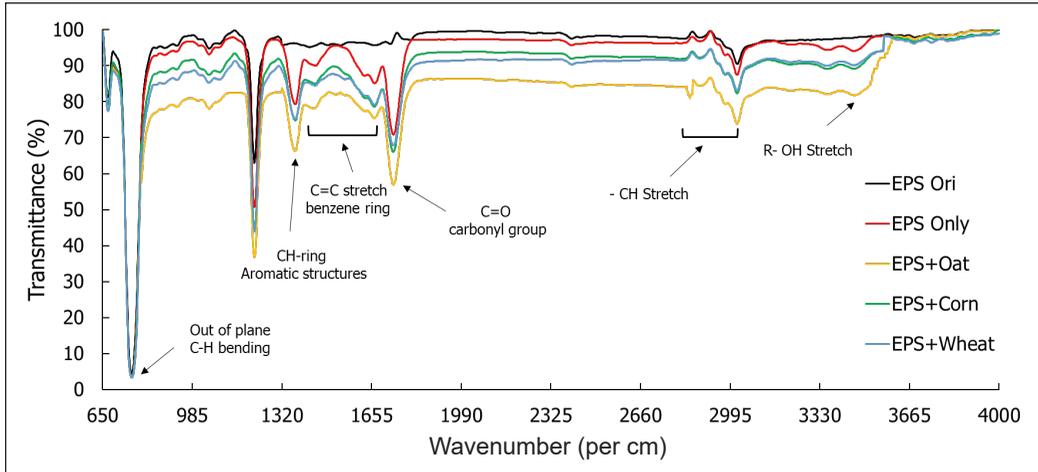


Figure 3. FTIR spectra of residual polymer (EPS group) from larva frass
Note. Fourier transform infrared spectroscopy analytical technique was used to characterize a major functional group of the degraded polymer after passing through the larva gut passage in the range of 650 - 4,000 cm^{-1}

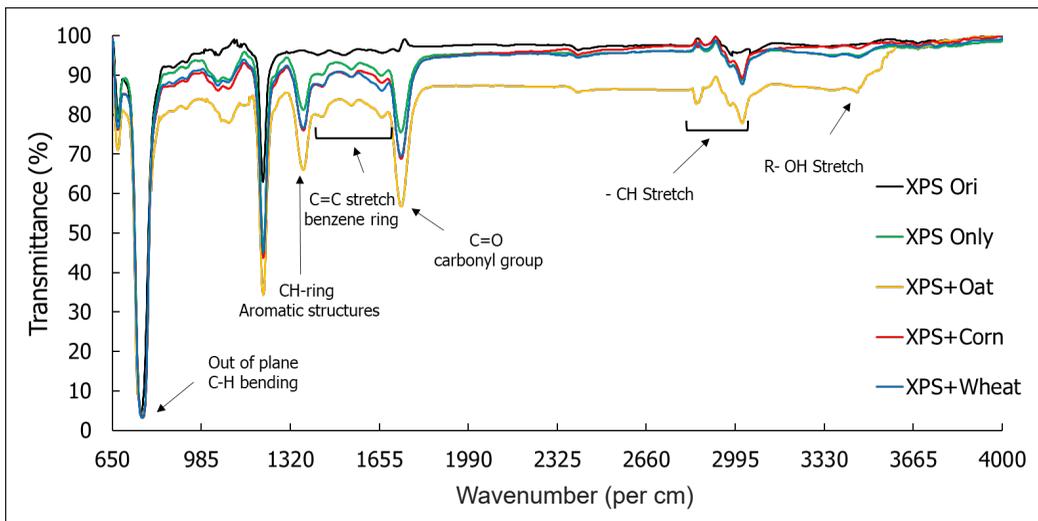


Figure 4. FTIR spectra of residual polymer (XPS group) from larval frass
Note. Fourier transform infrared spectroscopy analytical technique was used to characterize a major functional group of the degraded polymer after passing through the larva gut passage in the range of 650–4,000 per cm

of oxidation of ingested EPS (Brandon et al., 2018; Peng et al., 2020; Yang, Brandon, et al., 2018). The new peak appears at 1,369 per cm (CH- ring) in all EPS treatments assigned to the aromatic structures, as proved by Georgakopoulos (2003) and Yang, Brandon, et al. (2018). In the frass sample, a new absorption peak formed about 3,330–3,665 per cm. A new absorption peak ascribed to the hydroxyl group with OH stretch showed a change in surface characteristics from hydrophobic to hydrophilic (L. Yang et al., 2021). Overall, the

most effective EPS degradation by *Z. atratus* larva is treatment EPS oatmeal, followed by EPS (wheat bran), EPS (cornmeal), and weaker EPS alone based on the new peak appearance and level of intensities on peak decrease. The spectrum in the XPS group showed similar results as in the EPS group.

The aromatic structure and C=C stretch benzene ring were attributed to the peaks around per 1,360 cm (Yang, Brandon, et al., 2018). This peak appeared in all XPS treatments, while treatment XPS (oatmeal) showed a high peak indicating strong benzene ring cleavage. Besides, new peaks appeared around 1,160 and 1,710 per cm in all treatments, indicating the existence of C-O stretch and C=O carbonyl (J. Yang et al., 2014). The C-O stretch and C=O carbonyl on the polystyrene demonstrated that oxidation occurred, and deterioration was verified. The peak density showed that treatment XPS (oatmeal) is more distinct while treatment XPS (only) is the least. A peak was found at 2,839–3,000 per cm with distinct decreasing intensities. These peaks indicate that the C-H stretch was weaker, causing increasing vibration and instability, leading to the breakage of the C-H bond (Herman et al., 2015). Besides that, another new peak occurred around 3,330 per cm in all treatments attributed to the O-H stretch known as the hydroxyl band (S.-S. Yang et al., 2021). This peak indicates that the surface of XPS is changed to a hydrophilic character (Peng et al., 2019). EPS and XPS groups showed similar results, with the peak occurrence nearly identical, but each peak's intensity varies. The results showed

that treatment with oatmeal as a co-diet demonstrated the most effective in helping larvae degrade EPS and XPS based on the occurrence of new peaks and intensity levels of the related peaks.

The proton nuclear magnetic resonance (^1H NMR) analysis was used to prove the chemical changes in the residual polymer (Kundungal et al., 2021). Brandon et al. (2018) characterized residual PS versus original PS foam using ^1H NMR. A comparison of ^1H NMR spectra among residual EPS and XPS treatment was examined by extracting the residual polymer with chloroform-D. The number of new peaks increased, indicating that the chemical structure of polystyrene has altered, resulting in the formation of new chemical bonds between functional groups (Sekhar et al., 2016). In the EPS group (Figure 5), new peaks occurred in regions of 2.3 ppm, denoting the formation of C=O (carbonyl group), 2.8 ppm, 3.8 ppm, 4.2 ppm denoting the formation of -OH (hydroxyl group), while 5.1 ppm and 5.5 ppm known as CH=CH- (alkenyl hydrogen) (Liu, 2021; Yang, Brandon, et al., 2018). The C=O (carbonyl group) and -OH (hydroxyl group) indicated that EPS oxidation occurs, and the surface is readily hydrophilic. The CH=CH- (alkenyl hydrogen) indicates a hydrogen shift to double-bond carbon, where the chemical shift is likely to occur (Liu, 2021). It was noticed that treatment EPS (oatmeal) appeared to have more peaks when compared to EPS origin, indicating the highest effectiveness on EPS degradation and depolymerization. The effectiveness

of EPS depolymerization starts from most effective to less with EPS (oatmeal), EPS (wheat bran), EPS (cornmeal), then EPS (only). In the XPS group (Figure 6), new peaks occurred among treatments with 2.3 ppm, 3.8 ppm, 4.6 ppm, and 5.1 ppm compared with XPS origin. The peaks in 2.3 ppm are C=O (carbonyl group), 3.8 ppm, and 4.6 ppm denoting the formation of -OH, while 5.1 ppm is CH=CH-. In the XPS group, XPS (oatmeal) treatment showed more peaks than XPS origin. It

means that treatment XPS (oatmeal) is more effective in transforming and modifying XPS within the gut of the *Z. atratus* larva. Meanwhile, treatment XPS (wheat bran) and XPS (cornmeal) have more peaks than treatment XPS only when compared with XPS origin. The *Z. atratus* larva co-diet with wheat bran and cornmeal showed significant depolymerized XPS. EPS and XPS degradation are from most effective to less, such as oatmeal, wheat bran, cornmeal, and the sole diet.

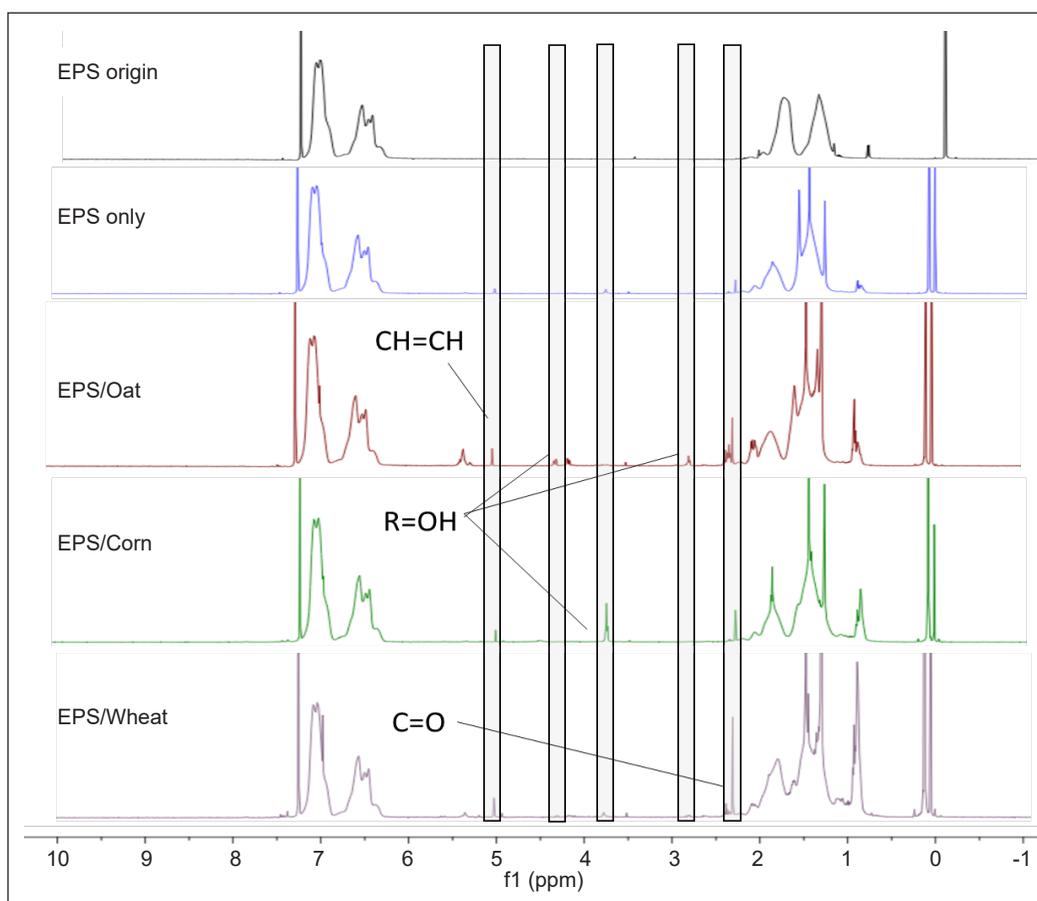


Figure 5. ¹H NMR spectra of residual polymer from larva frass (EPS group)

Note. ¹H nuclear magnetic resonance analytical technique was used to characterize changes in the end groups of the degraded polymer after passing through the larva gut passage

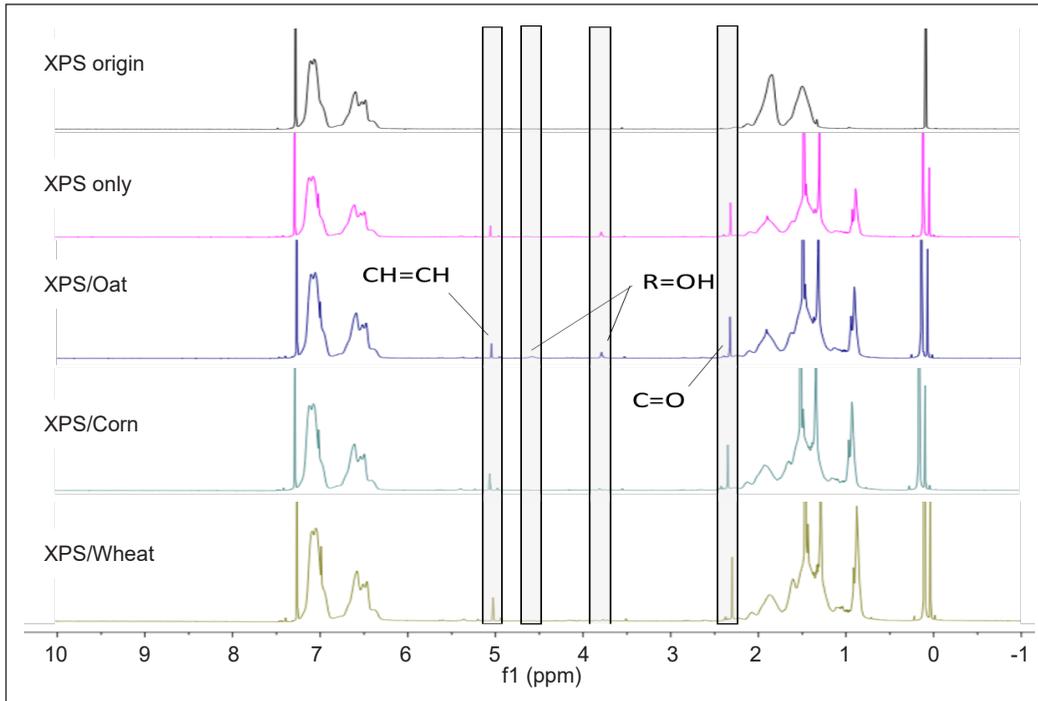


Figure 6. ^1H NMR spectra of residual polymer from larva frass (XPS group)

Note. ^1H nuclear magnetic resonance analytical technique was used to characterize changes in the end groups of the degraded polymer after passing through the larva gut passage

Growth and Development of *Z. atratus* Larvae After Ingested EPS and XPS Polystyrene

The growth and development of larvae were recorded throughout the study to compare the differences in *Z. atratus* larva growth after consuming EPS and XPS in different feeding diets. The *Z. atratus* larvae's growth and development depended on the types of diet and quantity of nutritional contents obtained. The final larvae length of *Z. atratus* larva among treatments revealed a significant difference ($p < 0.05$) with different diets (Figure 7). The results showed that EPS (oatmeal), EPS (wheat bran), and EPS (cornmeal) produced longer larva lengths with 48.01 ± 3.32 , 39.01 ± 2.29 , 30.03

± 1.36 mm, respectively, as compared to treatment EPS alone (26.10 ± 1.16 mm). In the XPS group, the larva's most extended final body length was attributed to treatment oatmeal as co-diet with 42.44 ± 2.50 mm, while the shortest was found in treatment XPS alone (24.87 ± 3.01 mm).

Oatmeal, wheat bran, and cornmeal provide essential nutrients for *Z. atratus* growth. These meals are protein-rich essentially for larvae growth (Emaleku et al., 2018; Onwulata et al., 2010; Rasane et al., 2015). Thus, all treatments in EPS and XPS groups containing supplements showed enhancement in final larva length compared to EPS or XPS alone. Larvae cuticles play an important role in larvae body elongation.

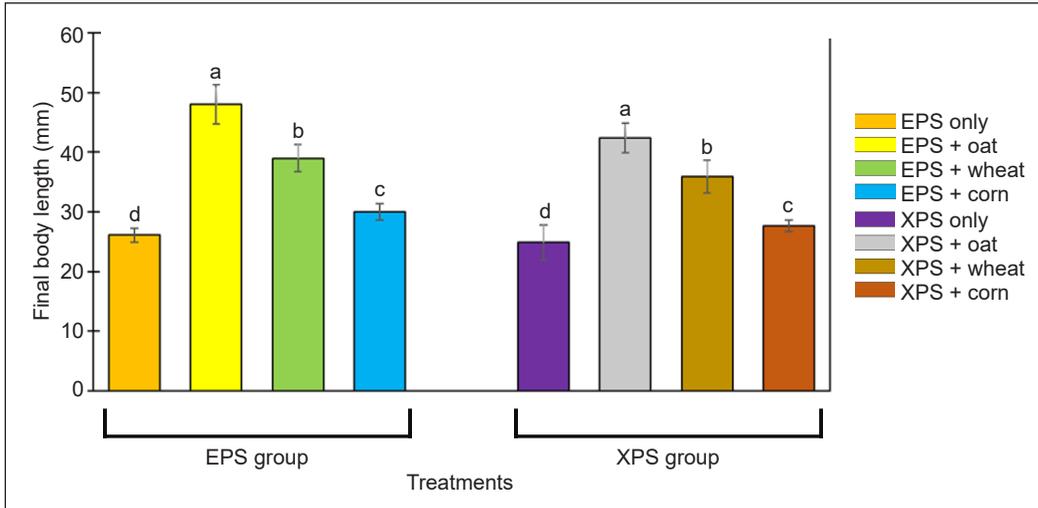


Figure 7. The final body length of the larva in each treatment with EPS and XPS groups. A digital vernier caliper was used to measure the larval body length

Note. All values were obtained from the mean of sixty replications with larvae reared separately per replication. Mean followed by different letters are significantly different at $p < 0.05$ level according to Tukey's one-way ANOVA. The same letter above the bar within the group represents no significant difference between treatments

Protein is one of the components in larvae cuticle (Kaleka et al., 2019). Oatmeal and wheat bran-rich protein compared to cornmeal indicated larvae growth was more vigorous in a protein-rich meal. Besides, oatmeal could alter the gut microbiota to enhance digestion activity, improving nutrient uptake (Kristek et al., 2018). The larva length in oatmeal treatment as a co-diet showed a significant and highest value in the EPS and XPS groups. Meanwhile, wheat bran and cornmeal showed less efficiency in promoting *Z. atratus* larva growth than oatmeal. Treatment with EPS alone (EPS group) and XPS alone (XPS group) had shown the shortest larva length. Miček et al. (2021) mentioned that the nutritional value of polystyrene-made materials is negligible but can convert into lipids after mineralization by providing a limited energy

source for larvae growth. Thus, EPS and XPS made from polystyrene only provided limited nutrition to *Z. atratus* larvae growth.

The total number of larval instars was counted when the *Z. atratus* larva exuvium was observed, and larva instars counting stopped when the larva started to curl (Kim et al., 2015). In EPS and XPS groups, the analysis showed a significant difference in the total number of larval instars among treatments (Figure 8). In the EPS group, treatment with EPS only showed the highest number of instars (20 ± 2 instars), followed by EPS (cornmeal) with 18 ± 1 instars, then EPS (wheat bran) with 16 ± 1 instars, and the lowest was EPS (oatmeal) with 15 ± 2 instars. Meanwhile, treatment with XPS only (20 ± 1 instars) showed the highest number of larval instars among the XPS group, followed by XPS (cornmeal) with

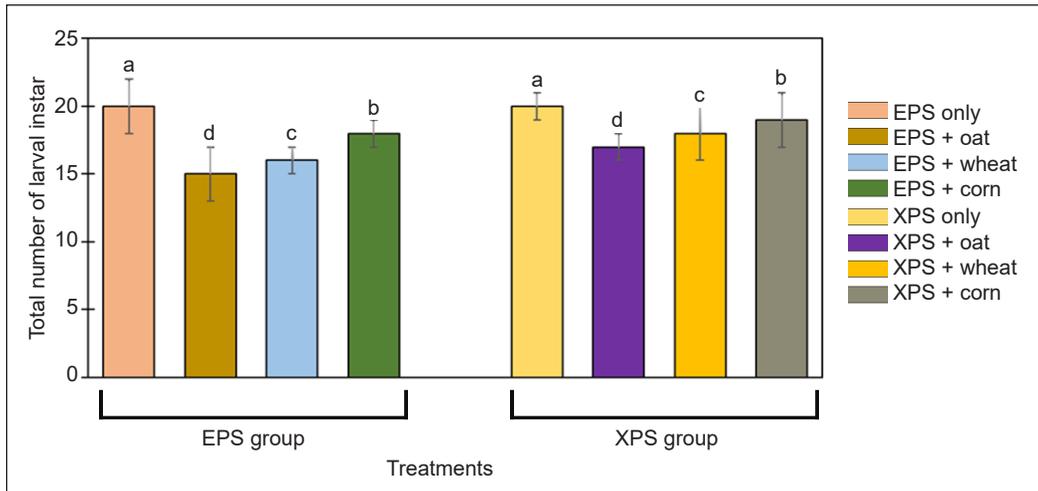


Figure 8. The total number of larval instars in each treatment with EPS and XPS groups. Each time of larvae molt (exuvium) was counted as a larval instar

Note. All values were obtained from the mean of sixty replications with larvae reared separately per replication. Mean followed by different letters are significantly different at $p < 0.05$ level according to Tukey's one-way ANOVA. The same letter above the bar within the group represents no significant difference between treatments

19 ± 2 instars. XPS (wheat bran) with 18 ± 2 instars and XPS (oatmeal) with 17 ± 1 instars. Nutrient intake influences larva's instars (Ribeiro et al., 2018). The rising number of instars might be a lack of food or nutritional deficiencies (Esperk et al., 2007). Treatment with EPS only and XPS only showed the highest number of instars.

In contrast, treatment with supplements revealed a lower total number of instars in EPS and XPS groups. Oatmeal as a supplement showed the lowest number of instars in the EPS and XPS groups, followed by wheat bran and cornmeal. The total number of instars in the wheatmeal and cornmeal supplement treatments was shorter than in the EPS (only) and XPS (only). Protein is a critical component of all organisms' tissues and essential for growth and development (Ortiz et al., 2016).

Protein content increases and the instars number will reduce (Morales-Ramos et al., 2010). Oatmeal and wheat bran contain many essential nutrients, especially protein. However, the structural characteristics and protein fraction distribution are different. Oat protein is soluble in salt or water, while wheat protein is insoluble in salt solutions (Rasane et al., 2015). Thus, oat protein is more readily digested and dissolves completely in the larvae's gut than wheat. Besides, Chojnacka et al. (2021) study revealed that cornmeal contains an insufficient amount of amino acids compared to wheat diets which play an important role in assisting protein digestibility. Therefore, the highest number of larval instars is treatment EPS or XPS as sole diets followed by cornmeal, wheat bran, and oatmeal.

In EPS and XPS groups, the analysis showed a significant difference among treatments in the total duration of larval (Figure 9). Treatment with EPS only showed the most prolonged larval duration with 223 ± 9 days in the EPS group. Meanwhile, treatment with XPS only (237 ± 5 days) also showed similar results to the EPS group. In contrast, co-diet meal treatment revealed a shorter larval instar duration in EPS and XPS groups. Ruschioni et al. (2020)'s study on *T. molitor* revealed that protein and carbohydrates are the primary nutrients that affect larval development time. Rho and Lee (2022) discovered that increased dietary nutrition, such as protein and carbohydrates, shortened larval development time.

The total duration of larva in the treatment-contained supplements was shown to be shorter than treatment EPS (only) and XPS (only). The shortened development time of larvae growth in treatment supplements is due to the sufficient nutrient (protein and carbohydrates) and other micronutrients provided. Oonincx et al. (2019) study proved that sufficient protein diets able to reduce development time in *T. molitor* and black soldier flies (*Hermetia illucens*). Oatmeal as a co-diet showed the shortest instar duration in the EPS group (157 ± 6 days) and XPS group (168 ± 4 days). Oatmeal is rich in macronutrients and micronutrients and helps to improve the diversity of larvae gut microbiota, indirectly improving

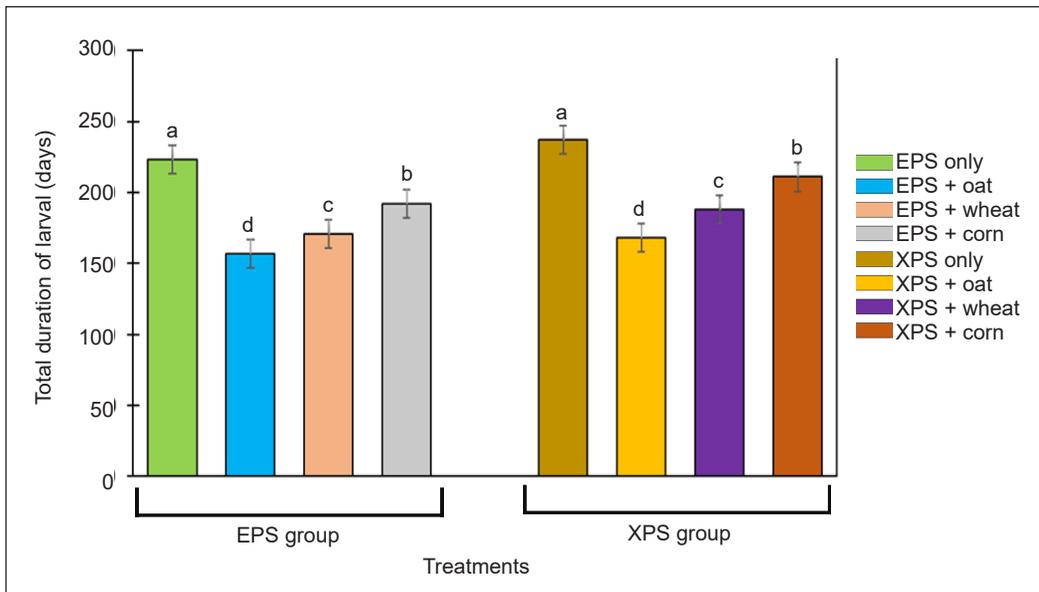


Figure 9. The total duration of larval in each treatment with EPS and XPS groups. Larvae curled were counted as the end of the larval stage

Note. All values were obtained from the mean of sixty replications with larvae reared separately per replication. Mean followed by different letters are significantly different at $p < 0.05$ level according to Tukey's one-way ANOVA. The same letter above the bar within the group represents no significant difference between treatments

nutrient uptake. Urbanek et al. (2020) study revealed that *T. molitor* consuming oatmeal increases the variety of gut flora. It indicated that oatmeal, wheat bran, and cornmeal, with sufficient nutrients, supplied, reduced larval development time.

Gut Microbial Analysis

Microbial community analysis was done on oatmeal treatment due to high EPS and XPS polystyrene consumption. The alpha analysis resulted revealed that treatment OB (EPS) showed the highest value in Chao1 indices, Fisher alpha index, and Shannon and Simpson index as compared to other diets such as OB (XPS) and OB (only). Chao1 and fisher alpha indices indicated that the richness of bacterial species is slightly higher in the diet of OB (EPS) compared to other diets. Shannon diversity and Simpson index refer to the species diversity and also showed the highest values in OB (EPS) groups followed by OB (XPS), then OB only (Table 2). Jiang et al. (2021) revealed that the difference in diversity might be due to dietary differences and related to gut ecophysiology. Study by Peng et al. (2019) on *T. molitor* feeding with polystyrene also showed increasing microbial community

diversity compared to normal diet feed. Therefore, the feeding meal that included EPS and XPS may lead the gut flora to grow, making it faster to digest. Similar phenomena were shown in four different diversity analyses where the treatment OB (EPS) and OB (XPS) showed higher value in terms of species richness and diversity compared to OB (only). Besides, density may affect the microbial population in the gut. High polymer density with high molar mass may cause microorganisms and oxidizing enzymes difficulty accessing polymer chains (Mohanani et al., 2020). Furthermore, XPS is denser than EPS, eventually reducing appetite and limiting the microbial population's growth. Thus, treatment OB (EPS) may have higher gut microbial diversity than OB (XPS).

The Bray-Curtis Distance dissimilarity measure was used to perform principal coordinate analysis (PCoA), which identified a cluster linked within treatment OB (only), OB (EPS), and OB (XPS). The microbiomes of *Z. atratus* larvae fed with treatment OB (EPS) and OB (XPS) were almost similar (Figure 10). However, the microbial community composition has differed from larvae fed between treatment OB (Only) and polystyrene (EPS and XPS) containing

Table 2

Bacterial diversity analyses based on illumine sequencing of the 16S rRNA gene amplicons for Zophobas atratus larvae under different diet conditions (OB only, OB + EPS groups, OB + XPS)

Samples	Chao1	Fisher	Shannon	Simpson
OB fed only	57.67	8.41	3.26	0.94
OB + EPS	66.00	9.89	3.62	0.96
OB + XPS	64.67	9.64	3.49	0.95

Note. OB = Oatmeal; EPS = Expanded polystyrene; XPS = Extruded polystyrene

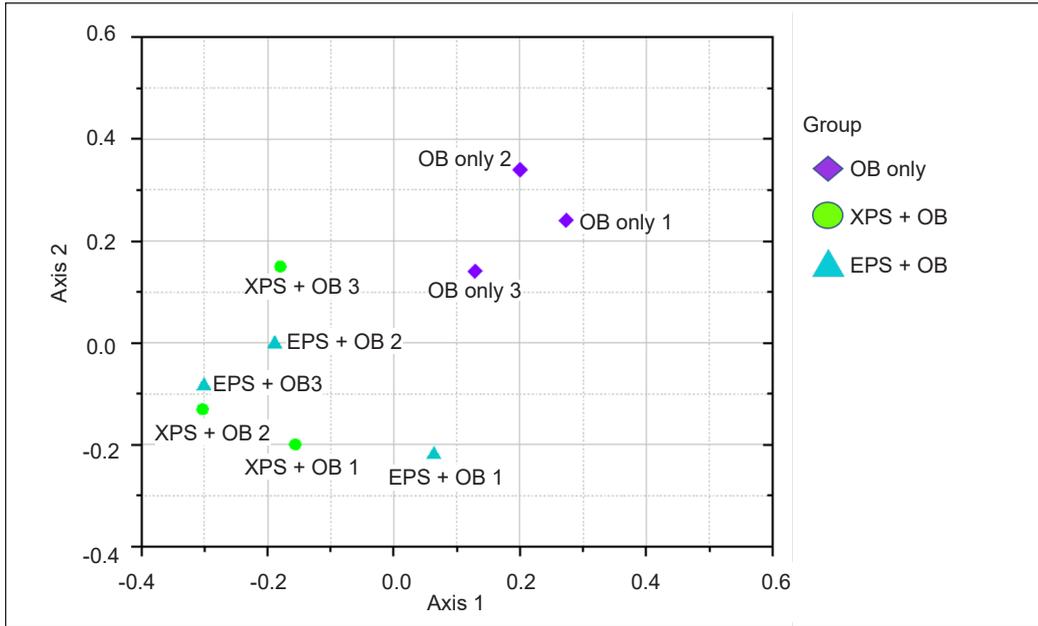


Figure 10. Analysis of gut microbiomes of *Zophobas atratus* larvae with PCoA
 Note. Principal coordinate analysis (PCoA) on the population of gut microbiomes of *Z. atratus* larvae between different diets (OB only, XPS + OB, and EPS + OB fed) based on Bray Curtis Distance. OB = oatmeal; XPS = Extruded polystyrene; EPS = Expanded polystyrene

diets. EPS and XPS are made from styrene monomer, but the manufacturing processing method, cell structure, and density differ. Thus, the diversity of microbial species between treatment OB (EPS) and OB (XPS) may have high similarities. Oatmeal provides essential nutrients for the growth of *Z. atratus* larva and improves the appetite of larvae to ingest polystyrene (Fu et al., 2020). Therefore, adding EPS and XPS with oatmeal help improve larvae appetite and digestion, indirectly shifting the composition of specific microbial species to digest the EPS and XPS. Lou et al. (2021) study on yellow mealworms with different diets revealed that the gut microbiome showed distinct clustering between PS + bran co-diet and normal feeding diets. Therefore, the microbial community was

distinct from treatment OB only compared to OB (EPS) and OB (XPS).

Relative abundance analysis was performed on the genus level. *Klebsiella* sp., *Kluyvera* sp., and *Enterobacter* sp. were discovered as OTUs with higher relative abundance in treatment OB (EPS) and OB (XPS) compared to OB (only) (Figure 11). These three OTUs were identified as facultative anaerobic bacteria at the genus level, meaning they can live in an environment with or without oxygen (Farmer III, 2015; Lopes et al., 2010; Maintinguer et al., 2017). *Klebsiella* sp. was a prominent genus in the gut microbiomes of *Z. atratus* larvae fed with treatment OB (XPS) and OB (EPS) with respective $8.49 \pm 5.60\%$ and $12.01 \pm 1.39\%$, which increased by 6.31% and 9.83 % compared to treatment

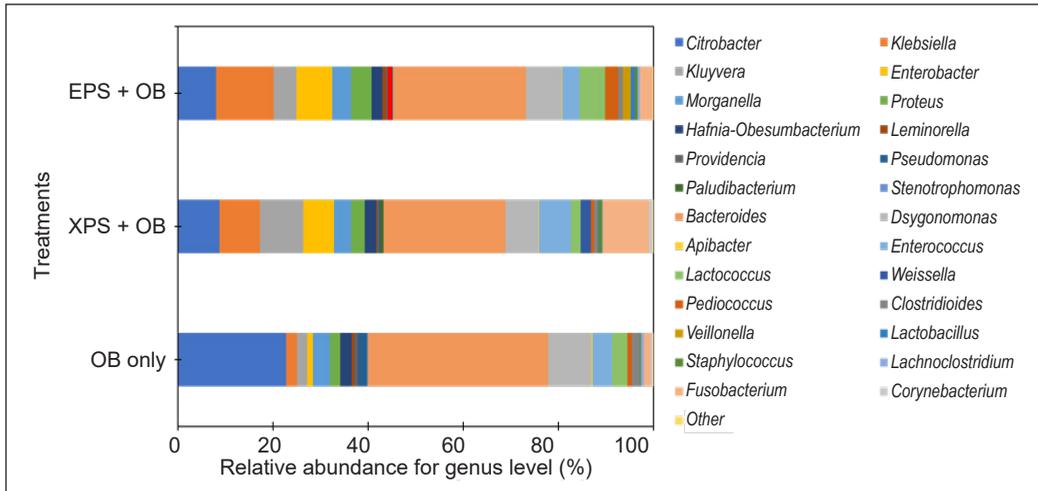


Figure 11. Analysis of gut microbiomes of *Zophobas atratus* larvae with relative abundance in genus level. Note. Relative abundance analysis on the population of gut microbiomes of *Z. atratus* larvae between different diets (OB only, XPS + OB, and EPS + OB fed) at the genus levels. OB = Oatmeal; XPS = Extruded polystyrene; EPS = Expanded polystyrene. All values represent mean \pm standard deviation

OB (only) with the relative abundance of $2.18 \pm 1.76\%$. *Klebsiella* sp. acts mutualistic relationship with insect hosts by digesting food in the gut and providing nutrients to the host (Sun et al., 2022). Besides, Machona et al. (2022) mentioned that *Klebsiella* sp. can degrade polystyrene in the gut system of *T. molitor*. Therefore, *Klebsiella* sp. showed higher abundance in OB (EPS), and OB (XPS) than OB (only) may be due to more *Klebsiella* sp. are require digesting oatmeal and EPS or XPS. Saygin and Baysal (2021) study revealed that *Klebsiella* sp. could degrade sub-microplastics through oxidation. The functional groups, such as the C=O carbonyl group, vinyl group, and oxygen-to-carbon ratio, were increased when the microplastics were treated by *Klebsiella* sp. Besides that, *Kluuyvera* sp., as one of the OTUs members in the microbial community of treatment OB (XPS) and OB (EPS), showed a shift to slightly higher

relative abundance ($9.10 \pm 1.23\%$ and $4.89 \pm 3.98\%$) respectively than treatment OB (only) with a relative abundance of $2.17 \pm 0.95\%$. *Kluuyvera* sp. can help *Z. morio* digest oatmeal diets and convert nutrients to the gut (Pivato et al., 2022).

Furthermore, XPS is a more closely packed structure than EPS, which takes time to digest (Turner, 2020). The relative abundance of *Kluuyvera* sp. is higher in OB (XPS) than OB (EPS) may be due to more need for this species among the gut community to digest XPS. Thus, *Kluuyvera* sp. might help degrade polystyrene in the gut of *Z. atratus* larval. *Enterobacter* sp. in treatment OB (XPS) and OB (EPS) with $6.46 \pm 4.95\%$ and $7.53 \pm 4.10\%$, which increased by 5.28% and 6.35% as compared to treatment OB (only) with a relative abundance of $1.18 \pm 0.22\%$. The symbiotic relationships between insects and *Enterobacter* species may be advantageous

to the host as it has varied capacities to catalyze nitrogen fixation, hydrolyze and ferment carbohydrates, and synthesize vitamins and pheromones for the host (Hendrichs et al., 2021). *Enterobacter* sp. needs a small amount to break down normal feeding diets (oatmeal) but adding EPS or XPS triggers rapid growth of *Enterobacter* sp., digested all the food sources. It also suggested that *Enterobacter* sp. can biodegrade food, including EPS and XPS. *Serratia marcescens* belonging to *Enterobacter* sp. showed overwhelming superiority in PS-fed gut microbiomes and was found in the PS-fed group (Lou et al., 2020). *Enterobacter* sp. should be investigated further since it is thought that various *Enterobacter* species may play a role in EPS and XPS polystyrene degradation. *Klebsiella* sp., *Kluyvera* sp., and *Enterobacter* sp. species in the genus were advised to be further validated by isolation and enzymatic testing to evaluate the capacity of plastic breakdown and the enzyme involved.

CONCLUSION

The results of this study demonstrated that superworms, *Zophobas atratus* larvae, from a Malaysian source could biodegrade EPS and XPS, supporting the ubiquity of PS degradation in this insect species. Furthermore, the PS consumption rate was enhanced significantly in the presence of co-diet with the sequence: oatmeal > wheat bran > cornmeal, positively depending on the protein content. FTIR and ¹H NMR analysis have further proven EPS and XPS polymer

biodegradation. Incorporating co-diets such as oatmeal, wheat bran, and cornmeal in EPS and XPS groups showed an increase in *Z. atratus* larva length, total number of instar, and duration of instar. *Kluyvera* sp., *Klebsiella* sp., and *Enterobacter* sp. in the gut of *Z. atratus* larvae were discovered to be strongly associated with the XPS and EPS polystyrene degradation. Thus, further studies are needed to verify the role of gut microbes in depolymerization and biodegradation via antibiotic suppression tests. Besides, further study on PS degradation rates can be done by examining THF extracted fraction.

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