Exploring the plastivorous activity of *Hermetia illucens* (Diptera Stratiomyidae) larvae

Linda Abenaim[1], David Mercati[2], Alessandro Mandoli[3], Joachim Carpentier[4], Grégoire Noël[4], Barbara Conti[1*], Rudy Caparros Megido[4], Romano Dallai[2]

[1] Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56126 Pisa, Italy
[2] Department of Life Science, University of Siena, Via Aldo Moro, 2, 53100 Siena, Italy
[3] Department of Chemistry and Industrial Chemistry, University of Pisa, Via Giuseppe Moruzzi, 13, 56124 Pisa, Italy
[4] Functional and Evolutionary Entomology, UR TERRA, Gembloux Agro-Bio Tech, University of Liège, Passage des Déportés 2, 5030 Gembloux, Belgique

*Correspondence: barbara.conti@unipi.it

Abstract:
Hermetia illucens (Diptera Stratiomyidae), also known as Black Soldier Fly (BSF), is one of the insect species most investigated for biodegradation ability in its larvae. *H. illucens* larvae can biodegrade organic waste but also contaminants like pesticides, antibiotics, and mycotoxins. This study wants to investigate the ability of these larvae to degrade polystyrene (PS). Experiments evaluated the growth performance, survival rates, intestinal and intracellular morphological alterations, degradation by-product formation and intestinal microbiota alterations of larvae fed a PS-enriched diet. Despite the addition of PS microparticles, no significant differences in growth or survival were observed compared to the standard diet (p>0.05). Confocal Laser Scanning Microscopy and Transmission Electron Microscopy confirmed the presence of PS microparticles in the larval gut, with potential signs of biodegradation. Metabolomic analyses identified styrene in the gut after 1 and 3 days of PS feeding, but its occurrence was likely due to thermal depolymerisation of the PS microparticles under GC-MS conditions. Metagenomic analysis revealed significant shifts in the intestinal microbiota. Notably, an enrichment of *Corynebacterium*, known for its role in aerobic PS degradation, and the abundance increase of other genera (*Enterococcus*, *Enterobacteriaceae*, *Enterobacter*, and *Escherichia-Shigella*) associated with synthetic polymer metabolism was observed. These results confirm the potential of BSF larvae to manage plastic waste through the interaction between their gut microbiota and synthetic materials. This study provides a foundation for future research focusing on isolating bacterial communities and enzymatic processes involved in polymer degradation, aiming to develop sustainable strategies for plastic waste management.

Keywords: Black Soldier Fly, polystyrene, microparticles, biodegradation, intestinal microbiota, by-products

1. Introduction

In recent years, research has increasingly focused on the ability of specific insect species to biodegrade and bioconvert food waste into valuable resources, such as chitin, proteins, lipids, vitamins, and other compounds. Nevertheless, some insects can detoxify and biodegrade contaminants and xenobiotic substances, including pesticides, mycotoxins, and antibiotics (Carpentier et al., 2024). The bioconversion, degradation, and detoxification processes carried out by insects are strongly influenced by their associated microbial communities, which are shaped by the insects' diet. As these communities adapt to the specific substrates consumed, bacteria specialised in digesting those substrates gain a competitive advantage within the gastrointestinal tract (Engel & Moran, 2013; De Smet et al., 2018; Carpentier et al., 2024). This adaptation is significant when dealing with environmental contaminants, like plastic, one of the most widespread and hazardous pollutants. In 2022, global plastic production reached 400.3 million tonnes, further intensifying the ecological challenge of plastic recycling (Plastics Europe, 2024). Among the various types of plastics, polystyrene (PS) which consists of styrene monomers, is a widely used plastic in different industrial applications, including food packaging (Block et al., 2017), and is considered one of the most polluting synthetic polymers (Ullah et al., 2023).

In the past five years, the use of insects for the biodegradation of synthetic polymers has gained significant attention in scientific literature. However, insects do not biodegrade plastics independently; instead, they rely on intestinal microorganisms and their digestive enzymes for this process (Brandon et al., 2018; Bulak et al., 2018; Cucini et al., 2020; Jiang et al., 2021; Arunrattiyakorn et al., 2022). These microorganisms produce enzymes that break down plastic polymer chains into smaller molecules, such as multimers and dimers, which can be transformed into carbon dioxide, methane, and water, adsorbed by microbial cells and used as a carbon source to fuel their energy cycles (Danso et al., 2019; Othaman et al., 2021). While not all insect species can completely biodegrade synthetic polymers, some species have shown the capacity to ingest and degrade different synthetic polymers, including polyethylene (PE), polystyrene (PS), polypropylene (PP), and polyvinyl chloride (PVC). Among these "plastivorous" species there are *Tenebrio molitor* Linnaeus, 1758 (Coleoptera Tenebrionidae), *Zophobas atratus* (Fabricius, 1775) (Coleoptera Tenebrionidae), *Alphitobius diaperinus* (Panzer, 1797) (Coleoptera Tenebrionidae), and *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera Pyralidae) (Sanchez-Hernandez, 2021; Kuan et al., 2022; Pivato et al., 2022; Noël et al., 2023). Although lepidopterans and coleopterans have demonstrated this potential, other insect species may represent alternative options worth exploring. An example is *Hermetia illucens* (Linnaeus, 1758) (Diptera Stratiomyidae), known as Black Soldier Fly (BSF), one of the most investigated insect species for the ability of its larvae to consume and degrade a wide variety of organic substrates, including waste (Nyakeri et al., 2017; Amrul et al., 2022), and convert them into valuable resources (Kaczor et al., 2022). Besides, recent research highlighted the ability of BSF larvae to degrade and reduce contaminants like pesticides, antibiotics, and mycotoxins (Camenzuli et al., 2018; Liu et al., 2020; Mei et al., 2022; Suo et al., 2023). However, recent studies also showed the capability of BSF larvae to chew and ingest microplastics of PVC, PE, PS, PP and PA (polyamide) without any apparent negative effects on their performance and survival (Cho et al., 2020; Romano & Fisher, 2021; Lievens et al., 2022; Piersanti et al. 2024; Wang et al., 2024; Heussler et al., 2024).

There is limited evidence that BSF larvae can degrade synthetic polymers, unlike other plastivorous insect species. Romano & Fisher (2021) found that BSF larvae exposed to PP microplastics exhibited high levels of propionic and butyric acid, which were absent in the larvae of the control diet. Since these short-chain fatty acids (SCFA) are of bacterial origin, their presence could be due to shifts in intestinal microbiota induced by the presence of plastic (Wang et al., 2021). Other authors observed changes in the intestinal microbiota of BSF larvae, suggesting a crucial role of the microbiota in degradation when exposed to plastic-contaminated diets (De Filippis et al., 2023, Pierasanti et al. 2024; Wang et al., 2024; Heussler et al., 2024). However, there are only a few studies on plastic biodegradation by *H. illucens*, so further and more in-depth investigations are needed.

This work aimed to investigate the ability of BSF larvae to consume and biodegrade a diet contaminated with PS microparticles. Using a multidisciplinary approach, we investigated the growth performance and survival rate of BSF larvae reared on the PS-contaminated substrate and the changes in the intestinal microbiota after administering a PS-contaminated diet.

2. Materials and methods

2.1. *Hermetia illucens* larval performance in a polystyrene-contaminated diet

Black Soldier Fly rearing is maintained under laboratory conditions (28 °C, 60% RH, and photoperiod 16:8 = L:D) at the Entomology Laboratory of the Department of Agriculture, Food and Environment at the University of Pisa since 2018 (Adamaki-Sotiraki et al., 2024). Thirty 6-day-old larvae were used to assess BSF larval performance, with five replicates per diet (150 larvae total). The larvae were starved for 24 hours, weighed, and measured before being placed in a 330 mL glass container with 30 g of the total diet. Two diets were used: a standard diet (poultry feed – Micheletti and Zei SRL,

Italy – soaked in water at a 40:60 ratio) and a polystyrene-contaminated diet (poultry feed soaked in water in the same ratio, mixed with a 2% aqueous solution of fluorescent PS microparticles). Before adding the poultry feed, the PS microparticle solution was uniformly distributed in the water and extensively mixed into the feed to ensure homogeneous dispersion. The PS microparticles were purchased from Sigma Aldrich, Merck KGaA (St. Louis, Missouri, United States), commercialised as latex beads, carboxylate-modified polystyrene, fluorescent yellow-green, with a mean diameter of 1.80-2.20 µm, a fluorescence of λex 470 nm and λem 505 nm, a density of 1.04-1.05 g/cm³, spherical shape and solid content in aqueous solution of 2.5% w/v. Given the use of 2% w/v aqueous PS solution with 2.5% w/v solid content, the total amount of solid PS in 30 g of diet was 15 mg, corresponding to a final concentration of 0.05% w/w (or 0.50 mg/g) of solid PS in the diet. Assuming a homogeneous distribution of PS in the food, each larva (30 larvae per diet) was estimated to ingest approximately 0.50 mg of PS microparticles, a concentration similar to that tested in previous studies (Lou et al., 2020; Wang et al., 2022). The microplastic concentration used in the present paper also falls within the range of microplastic levels in contaminated soil (0.03-6.7% w/w) and organic and food waste (0.001-5.6% w/w), as reported by Fuller and Gautam (2016) and Porterfield et al. (2023), which BSF larvae may naturally come into contact with.

The entire experiment, including five replicates per diet (150 larvae in total per diet), was repeated in three independent trials. Larval performances were assessed by recording survival rate (%), weight, and length every three days until the pupation. The weight and length of the larvae were measured when mature (15 days old). The individual weight of the larvae was assessed using an analytical balance, and their length was measured with a ruler. The larval survival rate (%) was calculated using the following formula:

$$\text{Larval survival rate (\%)} = \frac{\text{Final number of larvae}}{\text{Initial number of larvae}} \times 100$$

Before measurement, all larvae were rinsed with water and thoroughly dried with absorbent paper before weighing. As soon as prepupae were obtained, they were placed in a new box to allow pupation and track their survival and adult emergence.

2.2. Ultrastructural observations of *Hermetia illucens* larval gut

Ultrastructural observations of the larval gut were conducted using a Confocal Laser Scanning Microscope (CLSM; Zeiss LSM 700) at the Department of Life Sciences, University of Siena to assess the presence of PS in the larval gut and any morphological alterations. The observations were conducted on a total of 10 mature larvae, 5 treated and 5 control larvae of 15 days old, fed on the PS diet and standard diet respectively. Guts were dissected in sterile 0.1 M phosphate buffer (pH 7.2), to which 3% of sucrose was previously added (PB), under a stereomicroscope. Transmission Electron Microscopy (TEM) observations were performed to detect any intracellular differences between the midgut epithelium of larvae reared on the standard diet and those on the PS diet. The larval midgut epithelium was dissected and fixed at 4 °C in both treatments with 2.5% glutaraldehyde in PB. After 24 hours, each sample was rinsed in buffer and post-fixed in 1% osmium tetroxide for 1 hour at 4 °C. After dehydration in ethanol, the samples were incorporated in a mix of Epon-Araldite. Ultrathin sections of each sample were obtained with a Reichert Ultracut II E ultramicrotome, routinely stained with uranyl acetate and lead citrate, and then observed in a TEM Philips CM10 operating at an electron accelerating voltage of 80 kV.

2.3. Metabolomic analysis

To detect PS degradation products, 150 six-day-old larvae (divided into three replicates of 50 larvae each) were fed 50 g of the PS-based diet or 50 g of the standard diet. To assess the presence of PS degradation products, all larvae for each diet were collected after 1, 3, and 7 days, thoroughly washed, dried, and stored at -20 °C. Analytical samples for GC-MS

analysis were prepared according to Tsochatzi et al. (2021), by blending the larvae for 2 minutes with a domestic grinder and then subjecting them to lyophilisation (LIO 5PDGT, 5PASCAL) at -20 °C and 1030 mbar. After 24 hours of lyophilisation, the powder samples were stored at -20 °C until analysis. The extraction and GC-MS analyses were carried out at the Department of Chemistry and Industrial Chemistry at the University of Pisa. For the extraction, a lyophilised sample of 0.3-0.5 g was placed in a glass tube with 2.0 mL of dichloromethane (DCM). The tube was vortexed for 1 minute, sonicated for 1 hour at 25 °C, and centrifugated at 2500 rpm for 5 minutes at room temperature. The supernatant was filtered through a 0.2 mm PTFE (polytetrafluoroethylene) membrane and the clear filtrate was immediately subjected to analysis with a Varian 3800 GC coupled to a Saturn 2000 ion trap. The working conditions were: J&W DB-1MS column (30 m × 0.25 mm × 0.25 mm), 1.0 mL min^{-1} helium flow rate, splitless injection mode at 300 °C, with a purge flow of 1.5 minutes. The oven program started at 40 °C for 10 minutes, followed by a linear temperature ramp at 30 °C min^{-1} up to 320 °C. The electron ionization (EI+) spectra were acquired in the 55-350 *m/z* range in either the MS or MS/MS mode. Standard solutions of styrene (ST), a-methylstyrene (aMS), acetophenone (ACP), and cumyl alcohol (CA) in DCM (2-1000 ppb) were injected to determine the retention time and limit of detection (LOD) of each of the analyte reported by Tsochatzi et al. (2021).

2.4. Metagenomic analysis

Samples preparation and DNA extraction

DNA was extracted from the gut contents of 12 mature larvae (15 days old) per diet group (PS treated and control) for a total of 24 larvae at the Gembloux Agro-Bio Tech Department, University of Liège (Belgium). Before DNA extraction, the larvae were washed using distilled water and 70% ethanol for 10 minutes, followed by a triple rinse in distilled water. The specimens were killed in liquid nitrogen and dissected in an iced Petri dish with paraffin filled with PBS, to maintain cold conditions during the dissection and avoid degradation. Then, the entire gut dissected and extracted was placed into a sterile 1.5 mL Eppendorf and cryopreserved by directly plunging into liquid nitrogen, and the samples were kept at -80 °C. The RETSCH Mixer mill pulveriser was used to disaggregate the samples. The DNA was extracted using the Qiagen QIAamp PowerFecal Pro DNA Kit protocol, according to the manufacturer's instructions (ID: 51804, Qiagen, Hilden, Germany). The DNA quality was checked using a Nanodrop ND-10000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Qubit 1.0 (dsDNA quantitative analysis kit, Invitrogen, Carlsbad, CA, USA) was used to quantify the amount of DNA. The DNA integrity was evaluated by agarose gel electrophoresis (2%). The extracted DNA samples were stored at -20 °C until amplification.

16S rRNA gene amplification by Polymerase Chain Reaction

Polymerase chain reaction (PCR) amplification was performed on the V3-V4 hypervariable region of the 16S rRNA gene using primers pair S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATC-3') (Klindworth et al., 2013). All PCR reactions were performed in a volume of 50 µl containing 2 µl of BSF samples DNA, 1.5 µL of each primer at 1 µM, 20 µl of H_2O, and 25 µl of 2x KAPA Hifi HotStart Ready Mix (KAPA Biosystems, Wilmington, MA, USA). The PCR was carried out in a thermocycler by using the following cycle: 95 °C for 3 minutes, 98 °C for 20 seconds, 60 °C for 15 seconds, 72 °C for 30 seconds, 72 °C for 1 minute. The cycle was repeated 30 times. For quality control, 5 µL of PCR products were loaded in 2% electrophoresis gel and visualised under UV light to check if the expected band was obtained. The amplified products were purified using the NucleoSpin Gel and PCR Clean-up system (Macherey-Nagel, Düren, Germany). Then, the DNA amplicons were stored at 4 °C.

Gene library preparation and sequencing of the 16S rRNA

The PCR products were sent to the Institute of Biotechnology at Cornell University (Ithaca, NY, USA) for 16S library preparation and Illumina sequencing processing. Briefly, PCR products underwent purification through a PCR clean-up protocol which included the AMpure XP beads system (Beckman Coutler, Brea, CA, USA). Next, the NEXTERA XT Index Kit (Illumina, San Diego, USA) was utilised to affix dual indices and Illumina sequencing adapters for all the samples. DNA samples were standardised to a concentration of 4 nM and subsequently processed for sequencing in alignment with the Illumina system Denature and Dilute Libraries Guide. The MiSeq Illumina system was used for processing paired-end sequencing (2x250 bp).

Bioinformatics analyses

Subsequent demultiplexed raw sequences were processed using QIIME2 software (Quantitative Insights Into Microbial Ecology, version 2023.2) (Bolyen et al. 2019). Illumina Miseq sequencing of the V3-V4 region of the 16S rRNA gene generated a total of 2,430,269 reads (for both forward sequence count and reverse sequence count) across 12 samples from both, from a maximum of 147,091 to a minimum of 2,956, with an average of 101,261 ± 35,628 unpaired sequences per sample. The primers were trimmed with the plug-in cutadapt (Martin, 2011). After the reads were filtered on quality, denoised and merged with the DADA2 plug-in (Callahan et al., 2016) with --p-trunc-len-f 220, --p-trunc-len-r 220, --p-trim-left-f 18, --p-trim-left-r 21, resulting in high resolution of ASVs for downstream assessment. After filtering, 68,923 ± 26,151 unique sequences were conserved for the taxonomic assignation. The plug-in q2-feature-classifier (Bokulich et al., 2018) was used for the taxonomy assignment using a Naive Bayes classifier trained on the V3-V4 region of the target sequences and SILVA 138 reference database with a 99% similarity threshold (Robeson et al., 2021). Mitochondria and chloroplasts were excluded from the resulting ASVs table and then analysed using RStudio software (version 4.3.2) to visualise the different taxonomy between the two diets. To control for possible bias introduced by contaminants in our analyses, a filtering threshold of 1% for low abundant ASVs and additional filtering of ASVs present in less than 20% of samples were applied. The diversity analyses were measured with a q2-diversity plug-in following the core-metrics-phylogenetic method. In particular, the alpha diversity was based on the Faith Phylogenetic Diversity (Faith-pd) and Pielou-evenness metrics. The beta diversity was based on the Jaccard distance matrix and visualised with PCoA. The group significance between beta diversity was analysed using Permutational Multivariate Analysis of Variance (PERMANOVA). ASVs differentially abundant across groups were identified at genus and family levels with bias-corrected microbiome composition analysis (ANCOM-BC) (Lin & Peddada, 2020). Bacterial species with more than 1% relative abundance were included in this analysis to detect major species variations. Subsequently, the da-barplot was used to visualise the output of ANCOM-BC represented by the model Log Fold Change (LFC) which describes how much a quantity changes between an original and a subsequent measurement. The significance threshold, effect size threshold, and feature IDs were used to filter the results. Only the ASVs that passed all three filters were included in the output.

2.5. Statistical analysis

Larval growth performance (larval weight and length) was statistically compared between diets using the t-Student test (p <0.05) (JMP software, JMP®, Version 16. SAS Institute Inc., Cary, NC, 1989–2021). Alpha diversity of the microbiome data was assessed with the Kruskal-Wallis test followed by the Wilcoxon rank-sum test (p <0.05). Beta differences in overall microbiome taxonomic composition by diet were analysed using PERMANOVA on the Jaccard distance (p <0.05).

Differential abundance was evaluated with the FDR method (False Discovery Rate) incorporated in the ANCOM-BC with the Bonferroni method (p <0.05).

3. Results and Discussion

3.1. *Hermetia illucens* development in a polystyrene-contaminated diet

The weight and length of the larvae reared on the two different diets were checked every three days until all larvae were moulted in prepupae (18 days old). However, the comparison was made based on the larval performance shown in a previous paper (Adamaki-Sotiraki et al., 2024), when they were 15 days old (mature larvae), and their weight was maximum. At this time, in fact, the mature larvae stopped feeding to prepare for the moult and their weight decreased from then on (**Figures 1 and 2**).

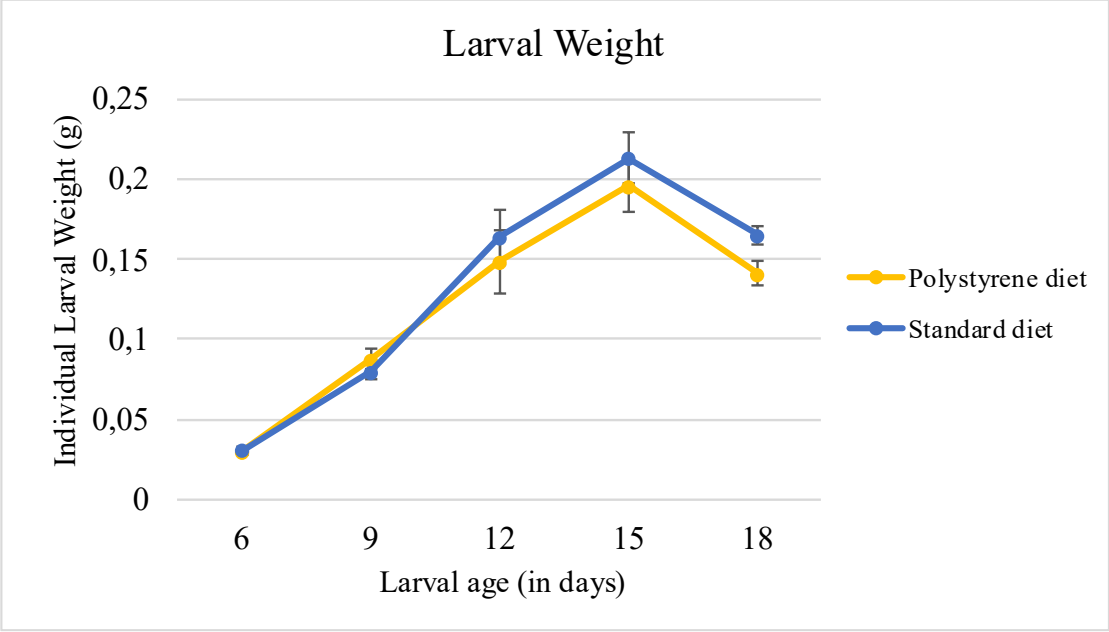


Figure 1. The average individual weight of *Hermetia illucens* larvae fed on the standard and polystyrene diet. A group of 30 larvae were tested, and 5 replicates for each diet were performed for 150 larvae/diet.

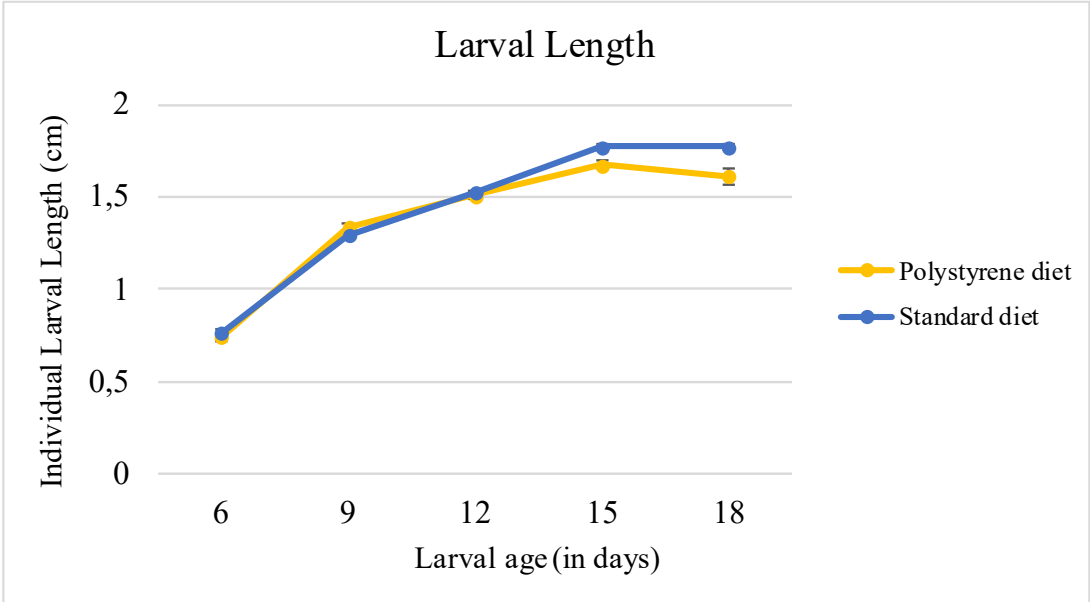


Figure 2. The average individual length of *Hermetia illucens* larvae fed on the standard and polystyrene diet. A group of 30 larvae were tested, and 5 replicates for each diet were performed for 150 larvae/diet.

The t-Student test did not show significant differences between the two diet treatments regarding individual larval weight and length ($t = 2.306$; $p = 0.528$ and $t = 2.306$; $p = 0.676$, for larval weight and length, respectively). The average individual larval weight was 0.190 ± 0.008 g for the PS diet and 0.210 ± 0.006 g for the standard diet. The average individual larval length was 1.670 ± 0.02 cm and 1.770 ± 0.01 cm for PS and standard diet, respectively.

No significant differences in larval survival rates (%) were observed between the two diets. Both groups of larvae exhibited high survival rates, with 98.8 ± 0.6% and 99.3 ± 0.6% for polystyrene and standard diets, respectively.

These results are consistent with previous research on the larval performance of plastivorous insects, such as *T. molitor*, *G. mellonella*, and *Z. atratus*, reared on the PS diet (Peng et al., 2020; Lou et al., 2020; Matyja et al., 2020; Yang et al., 2020; Quan et al., 2023). Similar outcomes have been reported when PS was supplied as the sole nutritional source (Peng et al., 2022; Jiang et al., 2021; Yang et al., 2020) and when PS was incorporated into the diet. Similarly to our study, PS added to bran did not affect the survival of *Z. atratus* larvae, which was approximately 94-95% with no significant differences compared to the control diet consisting of only bran (Peng et al., 2020). Our results also agree with studies on *G. mellonella* and *T. molitor* (Lou et al., 2020; Matyja et al., 2020). PS added to beeswax and bran diets showed no statistically significant differences in *G. mellonella* larval development when compared to control diets, while *T. molitor* larvae fed with PS added to the oats diet exhibited high survival rates (approximately 90%), with no significant differences compared to the corresponding control diet. However, in the studies previously described, plastic was provided in its original form, as the larval stages of the species used all possess mandibular mouthparts capable of chewing and ingesting plastic in this form.

The same type of microparticles used in the present study was also employed in other research, in which insect performances were assessed, even if in lower concentrations (Muhammad et al., 2021; Wang et al., 2021; El Kholy & Al Naggar, 2023). In particular, in *Bombyx mori* (Linnaeus, 1758) (Lepidoptera Bombycidae) larvae the exposition to PS microparticle in 10 µg/mL concentration (5–5.9 µm diameter) showed no significant changes in body mass or survival compared to the control group (Muhammad et al., 2021). In *Apis mellifera* (Linnaeus, 1758) (Hymenoptera Apidae), exposure to PS microparticles at concentrations of 0.5, 5 and 50 mg/L (25 µm diameter) resulted in only a small effect on mortality (from 1.43 to 1.6%), with no statistical difference in body weight compared to the control diet (Wang et al., 2021). In contrast, *Drosophila melanogaster* Meigen, 1830 (Diptera Drosophilidae) exposed to three different concentrations of PS microparticles (1.8-2.20 µm diameter), 0.005, 0.05 and 0.5 µg/ml, showed a significant reduction in survival compared to the control diet (El Kholy & Al Naggar, 2023).

In the case of BSF, our findings align with those of Cho et al. (2020), who reported no significant differences in larval weight between PS-contaminated and control diets, although the PS concentration in the treated diet was higher than in the present study (50, 100 and 200 mg/g). Once larvae were mature (15 days old), the average larval weight in their study was 0.2 g for both diets, which aligns closely with our results (0.190 ± 0.008 g and 0.210 ± 0.006 g, respectively for PS-contaminated and control diet). Moreover, the survival percentage for both diets was about 95%, like our results (98.8 ± 0.6% and 99.3 ± 0.6%, for PS and control diets, respectively). Besides PS, other synthetic polymers, such as PVC, PP and PE were also evaluated in the larval diet for BSF. Lievens et al. (2022) found no significant differences in weight (0.2 g) or survival rates (90%) between PVC-based (10 mg/g) and control diets, in line with our findings. Similarly, Romano and Fisher (2021) reported high larval survival (81%) with a PP-based diet, with no substantial differences compared to control diets, even though the concentration of microparticles in the treated diet was lower than the one in the present work (0.022 mg/g). Cho et al., (2020) also reported that BSF larvae reared on PE-based diet showed no significant differences in

survival rate or mean larval weight compared to the controls at all tested concentration (50, 100 and 200 mg/g). Our study confirms that including PS in the diet does not adversely affect BSF larval growth or survival. The data indicate that BSF larvae can grow similarly in environments contaminated with plastic. This capability is shared by other plastivorous species (*T. molitor*, *G. mellonella*, *Z. atratus*), further highlighting the potential of these organisms in bioconversion and plastic degradation research (Peng et al., 2020; Pena-Pascagaza et al., 2020).

3.2. Evidence of plastic presence in the larval gut

Confocal Laser Scanning Microscope (CLSM) observations of the guts of BSF mature larvae (15 days old) reared on the PS diet revealed the presence of fluorescent PS-microparticles inside the gut lumen (**Figure 3**), confirming the ingestion of food combined with the plastic particles.

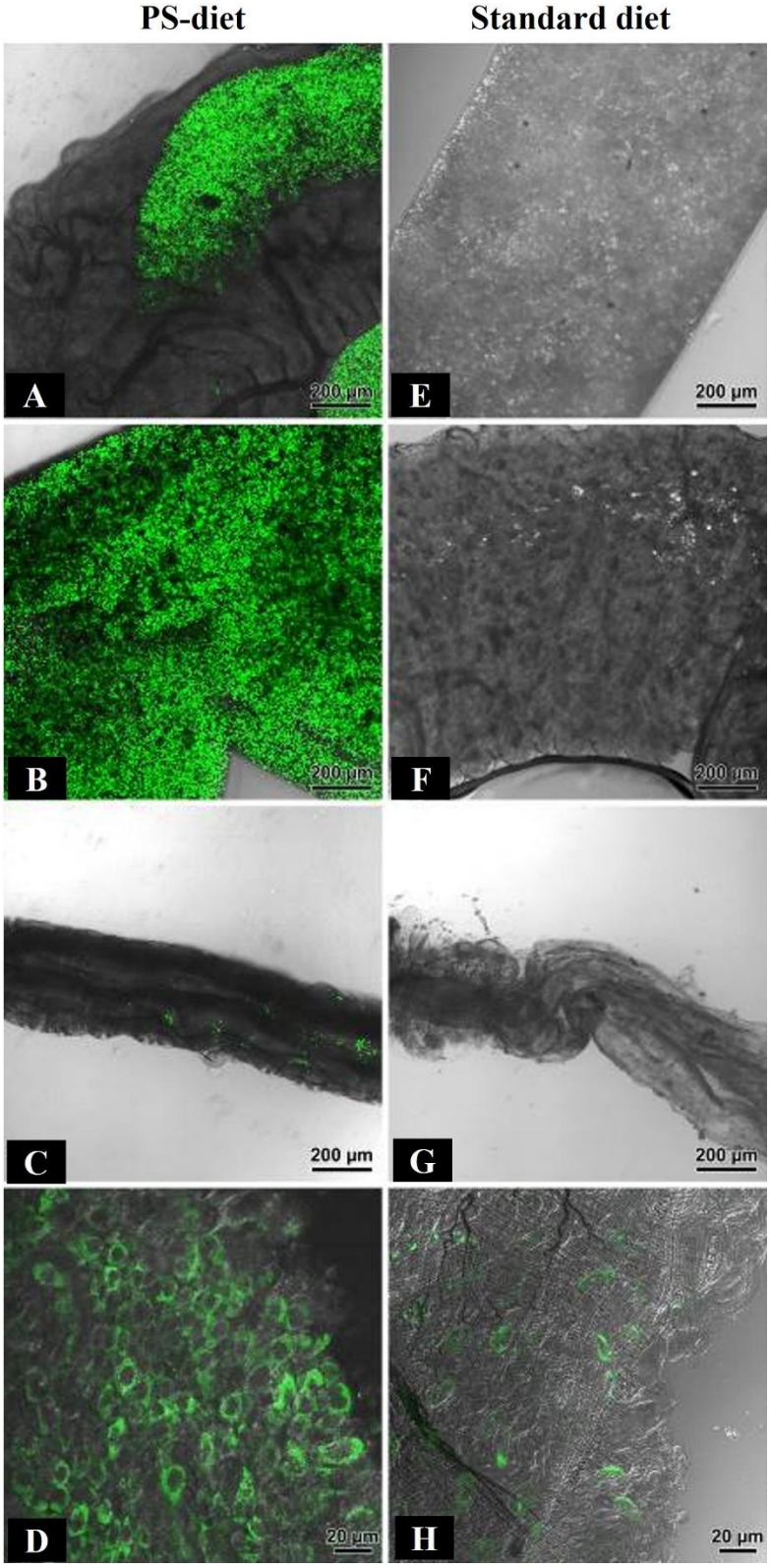


Figure 3. Confocal Laser Scanning Microscope observations of Black Soldier Fly larval gut. A-E) the foregut of larvae reared on both polystyrene diet and standard diet; B-F) the midgut of both treatments; C-G) the hindgut of both treatments; D-H) provides a detailed view of the midgut epithelium of larvae reared on both polystyrene diet (D) and standard diet (H).

The findings indicate that the foregut and midgut of BSF larvae fed a PS diet contained a high concentration of fluorescent PS microparticles (**Figure 3 A, B**). In contrast, the hindgut showed a significantly lower presence of PS microparticles,

as evidenced by the low fluorescence intensity (**Figure 3 C**). This latter effect may be related to the degradation of PS microparticles in the midgut due to the action of intestinal microbiota, as suggested by previous studies (Bruno et al., 2019). The midgut epithelial cells also exhibited strong fluorescence (**Figure 3 D**), implying that degraded PS fragments could have been transported across cells as metabolites or by-products of polymer degradation. Conversely, no fluorescence was detected along the gut of larvae fed the control diet (**Figure 3 E, F, G**). Nonetheless, some weak natural fluorescence was observed in the epithelial cells of the midgut of control-fed larvae, although with much lower intensity than the cells of larvae on the PS diet **(Figure 3 H**). This fluorescence is likely due to the intrinsic autofluorescence of the cells mainly originated from mitochondria and lysosomes, as described in prior studies (Monici, 2005; Koga et al., 2009). To further investigate the presence of PS microparticles at the cellular level, the midgut epithelium of BSF larvae reared on both diets was examined using TEM (**Figure 4**).

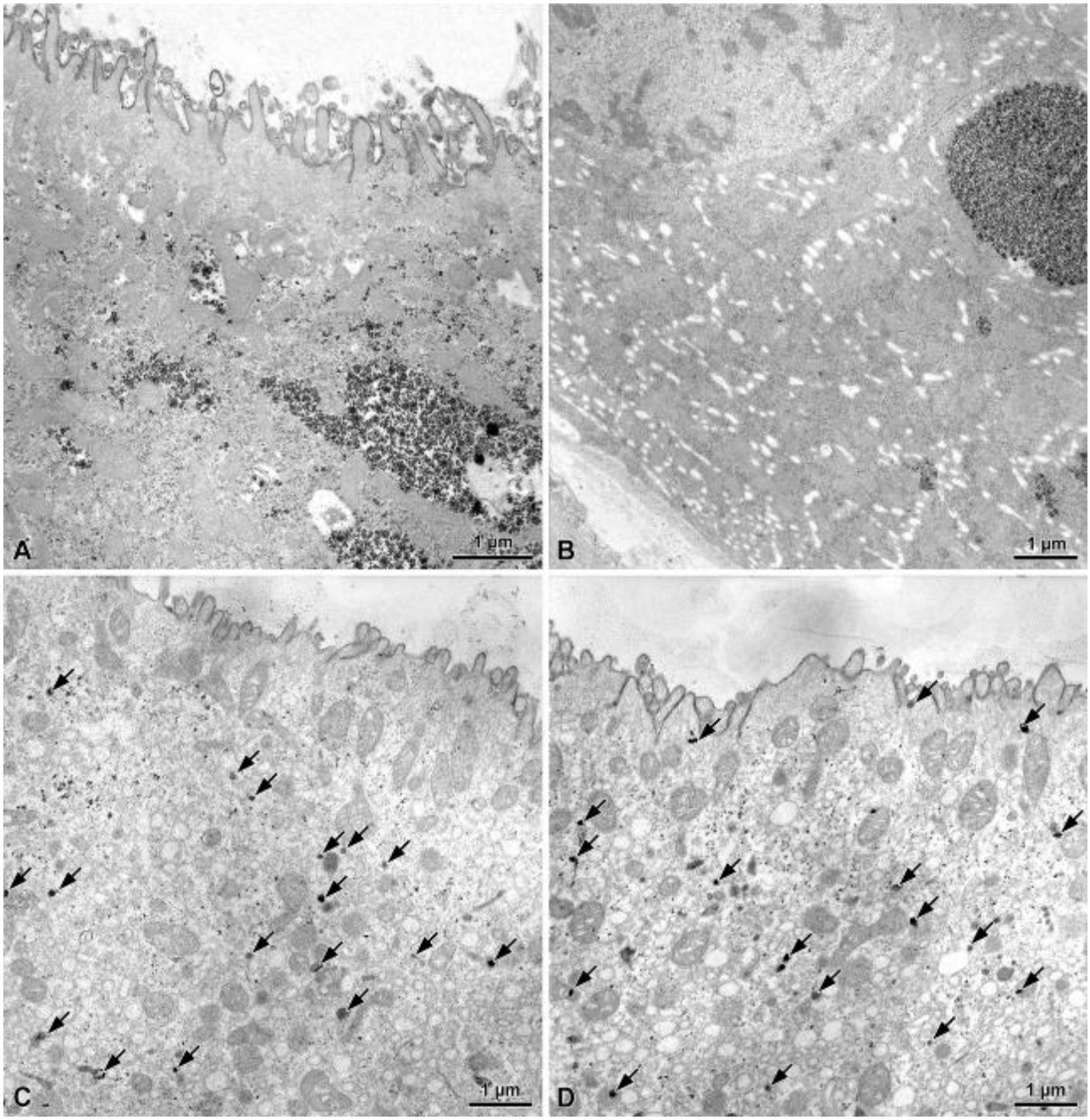


Figure 4. Transmission electron microscopy observations of the midgut epithelium of Black Soldier Fly larval gut. A) and B) Cross-sections of the midgut epithelium of larvae reared on the standard diet. C) and D) Cross-sections of the midgut epithelium of larvae reared on a diet contaminated with polystyrene. The arrows indicate the presence of numerous dense formations of approximately 50 nm in the epithelial cells, which are not present in those of larvae reared on the control diet.

TEM observations revealed that the midgut epithelium is characterised by cells approximately 20 µm in height. The cells of the midgut epithelium of larvae reared on the PS and control diets have a basal lamina thick 0.5-0.6 µm and a basal nucleus of approximately 2.3 µm in diameter. The basal region of the cells contains a series of deep invaginations of the plasma membrane, forming chains of small extracellular cavities that reach up to about half the height of the cell. In the apical region, the cells are separated by tortuous invaginations protruding into the cytoplasm, while the apex is bordered by a thin layer of microvilli about 0.6 µm high.

In the cells of insects reared on the control diet, numerous glycogen granules are visible in the apical and median regions, often compacted to form large clusters. In the cells of insects reared on the control PS diet, glycogen clusters are also observed, although they appear less thick. Dense formations of approximately 80-150 nm in diameter were detected in the apical region of the cells, just below the zone with microvilli. These formations were absent in the control and may be attributed to material derived from the degradation of PS.

Despite these formations, the morphology of the midgut epithelium in larvae exposed to the PS diet did not display significant alterations or detectable differences compared to those of the standard diet, following the results of Piersanti et al. (2024).

Confocal Laser Scanning Microscope investigations showed a high concentration of PS microparticles in the foregut and midgut, with fewer in the hindgut, suggesting potential biodegradation. Neither CLSM nor TEM revealed significant structural alterations in the larval intestine of BSF exposed to PS microparticles.

Previous studies by Wang et al. (2022) used fluorescently labelled PS microparticles to track their presence within the entire intestine of *G. mellonella* larvae. They reported a decrease in fluorescence over time (after 3, 6, 12 and 18h of 27, 56, 66, and 80%, respectively), suggesting the intestinal bacteria's potential depolymerisation and fragmentation of PS.

On the other hand, our TEM observations identified small, undefined dense aggregates in the midgut epithelial cells. Similarly, Wang et al. (2021) and Wang et al. (2022) observed similar formation via SEM in the midgut of *G. mellonella* and *A. mellifera.* These formations were linked to plastic particles, often accompanied by bacteria colonisation, indicating microbial degradation of plastics. For *G. mellonella*, PS particles in the midgut assumed irregular and smaller shapes after 6 hours from ingestion and were degraded entirely after 24 hours (Wang et al., 2022).

Our study's dense granules discovered in the epithelial cells likely represent by-products of PS decomposition and digestion. This hypothesis aligns with the findings by Elmekawy et al. (2023), who described similar formations in the epithelial cells of *G. mellonella* after exposure to low-density PE. Additional support comes from CLSM analysis, which revealed a high fluorescence level in the midgut epithelium. While commercially produced by an unspecified process, the fluorescent PS microparticles used in this study, likely contain fluorophores resistant to enzymatic attack and capable of intracellular transport (Prabhakar et al., 2020).

3.3. Evaluation of polystyrene biodegradation products

Previous work (Tsochatzis et al., 2020) identified DCM as a suitable solvent for extracting PS and its degradation products (monomers, oligomers, and oxygenated derivatives) from *T. molitor* larvae, thereby leading to the GC-MS identification of ST, aMS, ACP, CA, and a few ST oligomers as competent markers of PS biodegradation.

At variance with the literature investigation, in our study, GC-MS evidence could be gained only for the ST monomer (**Figure S1 a, b**) and just for the extracts of the larvae taken at one and three days. In this regard, it must be pointed out, however, that the injection of a diluted solution of PS latex in DCM also led to the ST peak being clearly observed in the chromatogram. This latter result is likely due to the thermal depolymerisation of PS in the hot GC-MS injection port (see for instance Yang et al. 2020), which makes ST ill-suited to assess biodegradation of the polymer material by this analytical technique.

Therefore, even if multiple studies have identified ST as the primary product in the early stages of microbial PS degradation (Othman et al., 2021; Tsochatzis et al., 2021; Wang et al., 2022), no definitive conclusion about this aspect can be drawn herein.

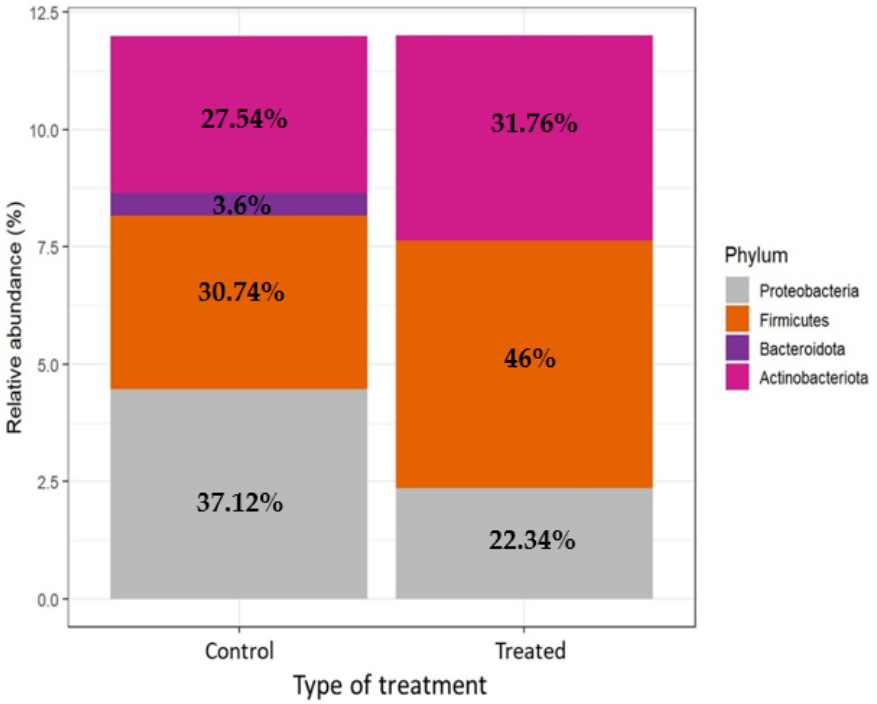
Moreover, the lack of observing the other low-molecular-weight substances noted above (aMS, ACP, CA) could reflect the occurrence of different biodegradation pathways depending on the microbial community involved (Warhurst & Fewson, 1994; O'Leary et al., 2002; Ho et al., 2018; Ru et al., 2020; Hou & Majumder, 2021; Wang et al., 2022).

However, it is worth pointing out that ST was detected only in larvae extracts at days 1 and 3 and was absent after 7 days (**Figure S2**). Although ST alone cannot be reliably used as a marker of PS degradation due to potential artefacts, its disappearance over time may suggest that the monomer and potentially other degradation products were metabolised or eliminate by the larvae .

3.4. Identification of polystyrene degradation microbial community

Microbial diversities were analysed in both experimental treatments after removing low-abundant ASVs (< 1%) in less than 20% of the samples. Following the filters, the number of ASVs decreased from 810 to 138. From the clustering of reads into ASVs and the annotation using the SILVA 138 database, Proteobacteria, Firmicutes, and Actinobacteriota were the main phyla observed for both treatments, representing 37.12 and 22.34%, 30.74 and 46%, and 27.54 and 31.76% for control and treated diet respectively (**Figure 5 a**). Additionally, the phylum Bacteroidota was found in small quantities in the gut microbiota of larvae reared on control diets (3.6%). **Figure 5 b** shows the average relative abundance of families represented by more than 1%. The most abundant families were Actinomycetaceae (31 and 29%), Enterococcaceae (16.4 and 38%), Morganellaceae (35.5 and 18%), Lactobacillaceae (9.4 and 6%), regardless of the treatment, and Corynebacteriaceae 5.7% only in the treated diet. Similarly, the most abundant genera were *Actinomyces* (31 and 29%), *Enterococcus* (16.4 and 38%), *Morganella* (25.5 and 8.5%), *Providencia* (10 and 9.3%), *Lactobacillus* (9.4 and 6%), and *Corynebacterium* (5.7% only in the treated diet) (**Figure 5 c**).

a) Phylum level of BSF bacterial community according to the diet (relative abundance >1%)

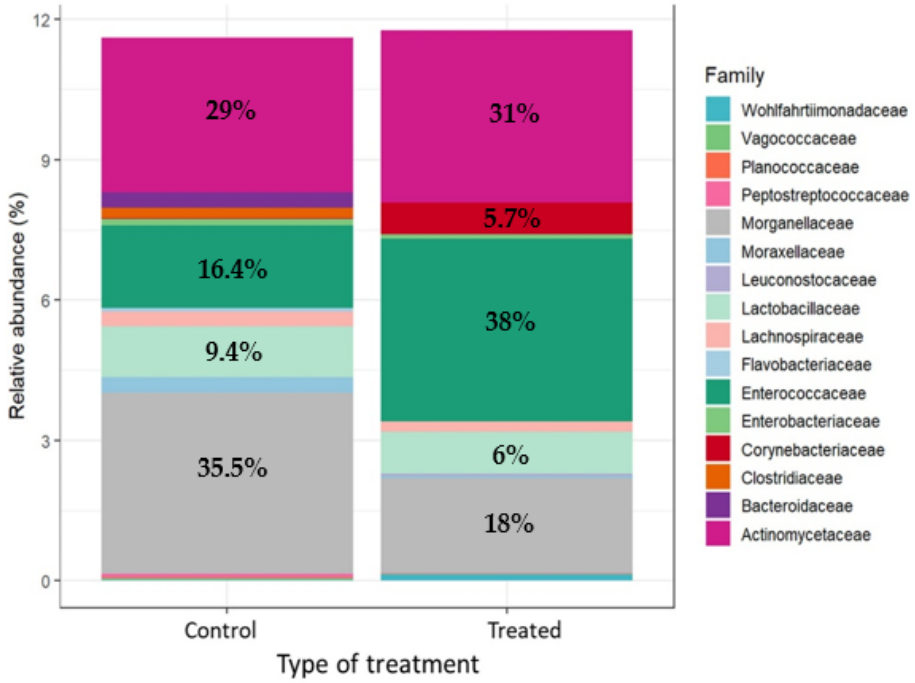


371

372

373

b) Family level of BSF bacterial community according to the diet (relative abundance >1%)



374

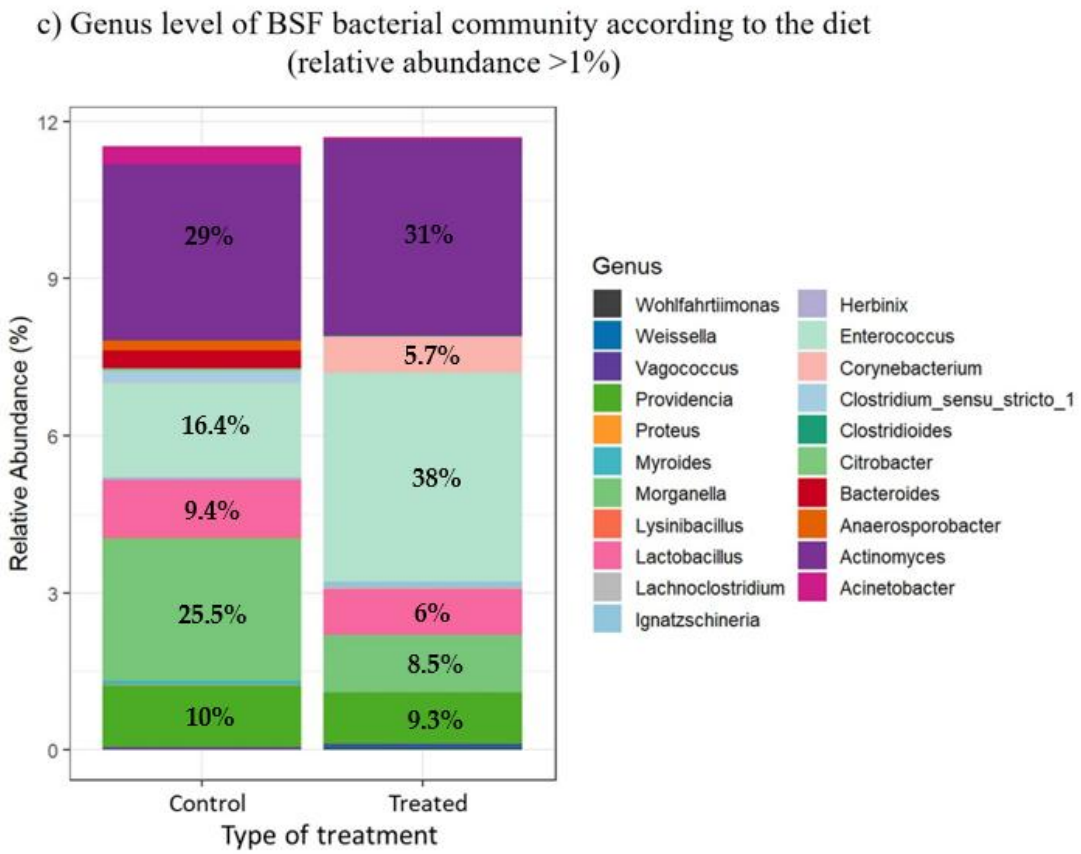


Figure 5. Taxonomic composition of the intestinal microbiota of *Hermetia illucens* at a) phylum level, b) family level, and c) genus level. Mean abundance was calculated for standard diet (control) (12 samples) and polystyrene diet (treated) (12 samples). Only bacteria taxa with an average relative abundance higher than 1% are reported.

The 16S rRNA gene sequencing evaluated the microbiota composition of BSF larvae fed with the PS-contaminated diet compared to the standard one. After only 2 weeks of feeding, the PS-containing substrate appeared to influence the composition of the intestinal bacterial community, compared to the control diet. At the phylum level, the PS-contaminated diet was predominantly composed of Firmicutes (46%), whereas in the control diet, the most abundant phyla were Proteobacteria (37.12%). On the family level, Actinomycetaceae exhibited similar abundance in the control and treated diets. However, Enterococcaceae was significantly more prevalent in the PS-contaminated diet, accounting for 38% of the total abundance. The Corynebacteriaceae family was present in the treated diet but absent in the control diet. At the genus level, the results were consistent; the PS-contaminated diet demonstrated a higher abundance of *Enterococcus* compared to the control, and the *Corynebacterium* genus was exclusively present in the PS-contaminated diet, comprising 5.7% of the relative abundance. These findings suggest that these microbial genera may play a role in polystyrene degradation.

The alpha and beta diversity metrics were analysed using the respective QIIME2 plugins for both treatments. The alpha diversity indexes, Faith's phylogenetic diversity and Pielou evenness, indicated no significant differences in both diets (see **Figure S3**). The Kruskal-Wallis test confirmed no statistically significant differences between the control and treated diet groups for both the qualitative measure of community richness in terms of phylogenetic relationships (Faith-pd; $H = 3.18$; $p = 0.07$) and the measure of community distribution equity (Pielou-evenness; $H = 2.18$; $p = 0.13$). These results suggest that neither treatment significantly affected the microbial communities' phylogenetic richness nor the distribution equity.

In contrast, beta diversity assessed by the Jaccard method and analysed with the PERMANOVA test showed a statistically significant difference ($F = 3.05$; $p < 0.001$) between the two treatment groups. This distinction is also visible in the PCoA graph for the Jaccard distance matrix (**Figure 6**), showing that the bacterial communities in the two treatments are dissimilar regarding their composition. This confirms the findings in **Figure 5 b** and **c**, which show different families and genera in the different treatments. This supports the hypothesis that the intestinal microbiota of the larvae fed on the PS-contaminated diet differs from that of the control diet due to the emergence of different microbial families and genera that can degrade the synthetic polymer.

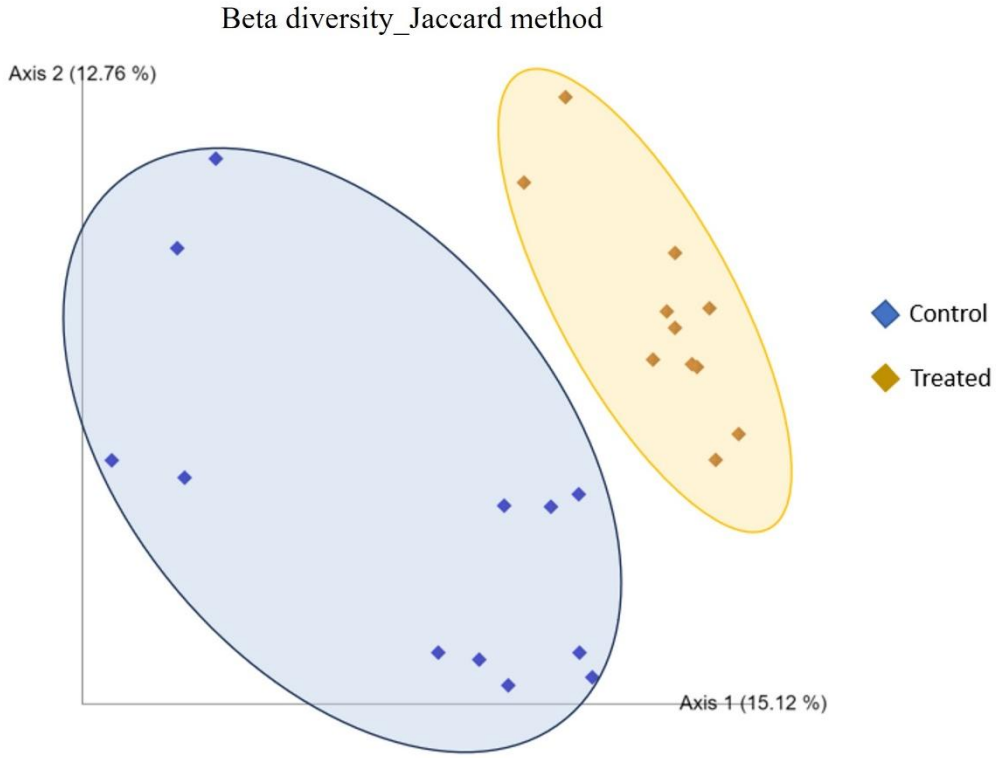
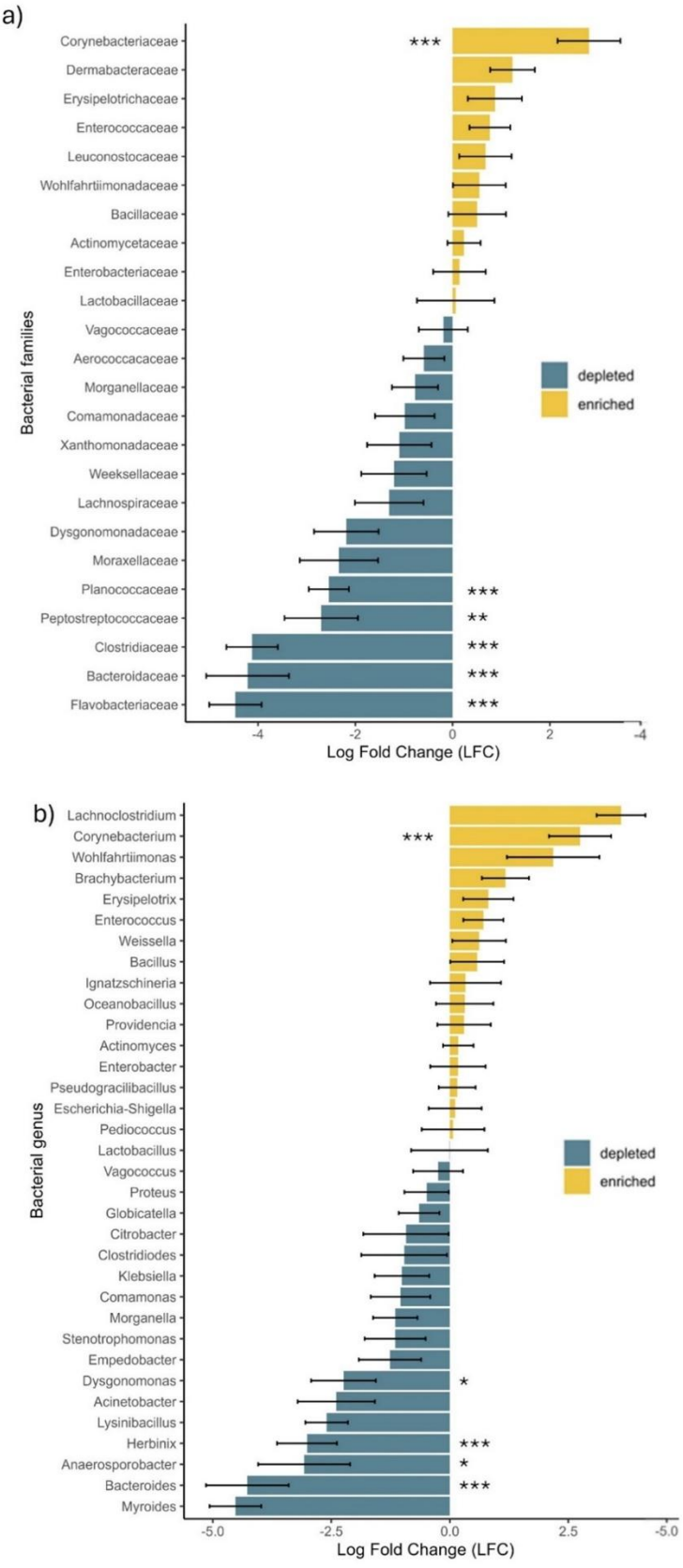


Figure 6. Principal Co-ordinates Analysis (PCoA) for the Jaccard distance metrics. There is no overlap between the distribution of intestinal microbial communities of the control and treated larvae, indicating the diversity between the two bacterial communities. Blue= control diet (standard diet); yellow= treated diet (polystyrene diet).

The PCoA plot showed a clear differentiation in the distribution of intestinal microbial communities between the two treatments, suggesting a relationship between microbial composition changes and polystyrene degradation; this is consistent with findings by De Filippis et al. (2023) and Wang et al. (2024). This distinction was further validated by examining the differential abundance of the various families and genera of bacteria found in both diets using ANCOM-BC (**Figure 7**).


a)
Corynebacteriaceae
Dermabacteraceae
Erysipelotrichaceae
Enterococcaceae
Leuconostocaceae
Wohlfahrtiimonadaceae
Bacillaceae
Actinomycetaceae
Enterobacteriaceae
Lactobacillaceae
Vagococcaceae
Aerococcacaceae
Morganellaceae
Comamonadaceae
Xanthomonadaceae
Weeksellaceae
Lachnospiraceae
Dysgonomonadaceae
Moraxellaceae
Planococcaceae
Peptostreptococcaceae
Clostridiaceae
Bacteroidaceae
Flavobacteriaceae
***
***
**
***
***
***
Bacterial families
depleted
enriched
-4
-2
0
2
-4
Log Fold Change (LFC)
b)
Lachnoclostridium
Corynebacterium
Wohlfahrtiimonas
Brachybacterium
Erysipelotrix
Enterococcus
Weissella
Bacillus
Ignatzschineria
Oceanobacillus
Providencia
Actinomyces
Enterobacter
Pseudogracilibacillus
Escherichia-Shigella
Pediococcus
Lactobacillus
Vagococcus
Proteus
Globicatella
Citrobacter
Clostridiodes
Klebsiella
Comamonas
Morganella
Stenotrophomonas
Empedobacter
Dysgonomonas
Acinetobacter
Lysinibacillus
Herbinix
Anaerosporobacter
Bacteroides
Myroides
***
*
***
*
***
Bacterial genus
depleted
enriched
-5.0
-2.5
0.0
2.5
-5.0
Log Fold Change (LFC)

417

Figure 7. Amplicon Sequence Variants are differentially abundant in the two treatments. The bars describe the relationship between the treated group and the control group. Yellow bars represent the Amplicon Sequence Variants more abundantly in the treated group. In contrast, blue bars represent the Amplicon Sequence Variants less abundantly in the treated group compared to the control one. Thus, some Amplicon Sequence Variants are enriched (yellow) and depleted (blue) in the treated group. The Amplicon Sequence Variants depleted in the treated group are enriched in the control group, and vice versa. Graphs a) represent the family level, and b) represent the genus level. The asterisks indicate statistical significance: *** maximum significance at 0.0001%, ** significance at 0.001%, * significance at 0.05%. The bars indicate the standard error (SE).

The differential abundance analyses revealed that the gut microbiota of BSF larvae fed with PS diet exhibited enrichment in several bacteria families, including Corynebacteriaceae, Demabactericeae, Erysipelotrichaceae, Enteroccocaceae, Leuconostocaceae, Wohlfahtiimonadaceae, Bacillaceae, Actinomycetaceae, Enterobacteriaceae, and Lactobacteriaceae. The family Corynebacteriaceae demonstrated a statistically significant differential abundance compared to the control diet. Among the bacterial genera enriched in the PS-fed larvae, *Lachnoclostridium*, *Corynebacterium*, *Wohlfahrtiimonas*, *Brachybacterium*, *Erysipelothrix*, *Enterococcus*, *Weissella*, *Bacillus*, *Ignatzschineria*, *Oceanobacillus*, *Providencia*, *Actinomyces*, *Enterobacter*, *Pseudogracilibacillus*, *Escherichia-Shigella*, and *Pediococcus* were identified, with *Corynebacterium* showing a particularly significant differential abundance. These findings are consistent with previous studies by Sun et al. (2022) and Peydaei et al. (2021), which reported a substantial increase in the genus *Corynebacterium* in larvae of *Z. atratus* and *G. mellonella* fed with PS diet. *Corynebacterium* is an aerobic bacterial genus known for its involvement in the degradation pathway of polystyrene (Itoh et al., 1996; Itoh et al., 1997; Itch et al., 1997; O'Leary et al., 2002; Mooney et al., 2006; Tischler, 2015; Danso et al., 2019). Specifically, *Corynebacterium* strains AC-5 and ST-5 can metabolise styrene via oxidation, converting it into styrene oxide and phenylacetaldehyde through the action of styrene monooxygenase and styrene oxide isomerase enzymes (Itoh et al., 1996; O'Leary et al., 2002; Tischler, 2015; Othman et al., 2021). This pathway was also observed by Sun et al. (2022), in which *Corynebacterium*, isolated from the intestinal microbiota of *Z. atratus* larvae, degraded PS effectively using these enzymes. Our findings strongly suggest that the exclusive presence of the genus *Corynebacterium* in the intestinal microbiota of PS-fed BSF larvae is closely associated with the biodegradation of polystyrene microparticles.

In addition to *Corynebacterium*, other bacterial species such as *Pseudomonas putida*, *Xanthobacter*, *Rhodococcus*, *P. fluorescens,* and *P. aeruginosa*, have been implicated in the polystyrene biodegradation pathway (Warhurst & Fewson, 1994; Itoh et al., 1996; Itoh et al., 1997; O'Leary et al., 2002; Mooney et al., 2006; Singh et al., 2012; Tischler, 2015; Danso et al., 2021; Othman et al., 2021). Examination of the gut microbiota of PS-fed insect species revealed an increase in bacteria associated with PS metabolisation. For instance, Jiang et al. (2021) observed that *Enterococcus*, *Enterobacteriaceae*, *Lactococcus*, *Enterobacter*, *Serratia*, and *Escherichia-Shigella* were favored in the gut microbiota of *Z. atratus*, *G. mellonella*, and *T. molitor* subjected to a PS diet. Similarly, our results demonstrated an increase in *Enterococcus*, *Enterobacteriaceae*, *Enterobacter*, and *Escherichia-Shigella* in the intestinal microbiota of PS-fed BSF larvae.

Furthermore, studies on plastic-consuming insects, such as Urbanek et al. (2020), Jiang et al. (2021), Sun et al. (2022), and De Filippis et al. (2023), have consistently reported an increase in the abundance of the phyla Actinobacteria and Firmicutes when exposed to PS. These findings align with our results and suggest a potential correlation between these phyla and their capacity to degrade or adapt to synthetic polymer-rich diets. Interestingly, despite the enrichment of specific bacterial taxa, the total bacterial abundance in the PS-fed larvae was lower compared to the control diet. This reduction could be attributed to developing chemical degradation by-products, such as styrene or styrene oligomers, which may exert a bacteriostatic effect, as proposed by Tsochatzi et al. (2021).

Our findings highlight the potential of the intestinal microbiota of BSF larvae for isolating microbial strains optimised for plastic degradation. Investigating the microbial communities present in the gut of BSF larvae reared on synthetic polymers, such as PS, could facilitate the identification of enzymes with applications in plastic waste management. Recent studies on BSF larvae raised on diets contaminated with other microplastics, such as PVC, PP, and PE, have also suggested interactions between the gut microbiota and synthetic polymer degradation (Piersanti et al., 2024; Wang et al., 2024). However, further research is essential to elucidate the mechanisms by which BSF larvae contribute to the degradation of synthetic polymers, particularly polystyrene. Comprehensive investigations into the microbiota and enzymatic processes involved are crucial to understanding the role of BSF in polymer degradation and optimising its application in bioremediation strategies.

4. Conclusions

The multidisciplinary investigation into the plastivorous activity of *H. illucens* larvae provides encouraging evidence of their capacity to degrade and bioconvert synthetic polymers, particularly polystyrene. The larval performance evaluation demonstrated remarkable adaptability, with no significant differences in growth performance or survival rates observed between larvae fed a PS-contaminated diet and those on a standard diet. These findings suggest that BSF larvae can utilise synthetic polymers as a carbon source and thrive in PS-rich environments. Ultrastructural observations through CLSM and TEM revealed the presence of PS microparticles primarily in the foregut and midgut, with minimal presence in the hindgut. Notably, fluorescence detected within the midgut epithelial cells suggests the translocation of PS by-products generated during microbial degradation. TEM analysis further confirmed dense aggregations of these by-products within the gut. Metagenomic analysis provided additional insights into the microbial communities associated with PS exposure. While we acknowledge that shift in gut microbiota composition can be influenced by a range of factors, our findings revealed changes compared to the control group that are consistent with those observed in other plastivorous insects. Specifically, the enrichment of *Corynebacterium* (a genus known for its role in aerobic PS degradation pathways) and the increased abundance of *Enterococcus*, *Enterobacteriaceae*, *Enterobacter*, and *Escherichia-Shigella* (bacterial taxa previously associated with plastic degradation), suggest a potential role in the bioconversion process. It is important to note that the PS microparticles used in this study were carboxylate-modified latex beads. Although these particles are widely used in laboratory settings as a model for polystyrene exposure, as previously reported, they differ from naturally occurring environmental microplastics, probably influencing microbial interactions or degradation dynamics. However, their use was essential in this and similar studies as their small size and aqueous dispersion made them the only viable option to ensure ingestion by *H. illucens* larvae, whose mouthparts are adapted to semi-liquid substrates. Moreover, their fluorescent properties enabled precise visualisation during ultrastructural observations.

This study strengthens the hypothesis that BSF larvae contribute to plastic waste degradation, elucidates some mechanisms underlying this process, and provides a valuable foundation for future research on insect-mediated polymer degradation. Future studies should focus on isolating and cultivating the bacterial communities involved and elucidating the enzymatic pathways driving PS biodegradation. These investigations will enhance the understanding of BSF larvae's potential in sustainable waste management applications, offering a promising strategy to address the global challenge of plastic pollution.

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Author contributions: Conceptualisation: Abenaim Linda**;** Investigation: Abenaim Linda, Mercati David, Mandoli Alessandro; **Methodology:** Abenaim Linda, Mercati David, Mandoli Alessandro, Carpentier Joachim, Noël Grégoire; **Data curation:** Abenaim Linda; Formal analysis: Abenaim Linda, **Writing-original draft preparation:** Abenaim Linda; **Writing- review and editing:** Abenaim Linda, Mercati David, Mandoli Alessandro, Carpentier Joachim, Noël Grégoire, Conti Barbara, Caparros Megido Rudy, Dallai Romano; **Supervision:** Conti Barbara, Caparros Megido Rudy, Dallai Romano; **Funding acquisition:** Conti Barbara, Caparros Megido Rudy, Dallai Romano.

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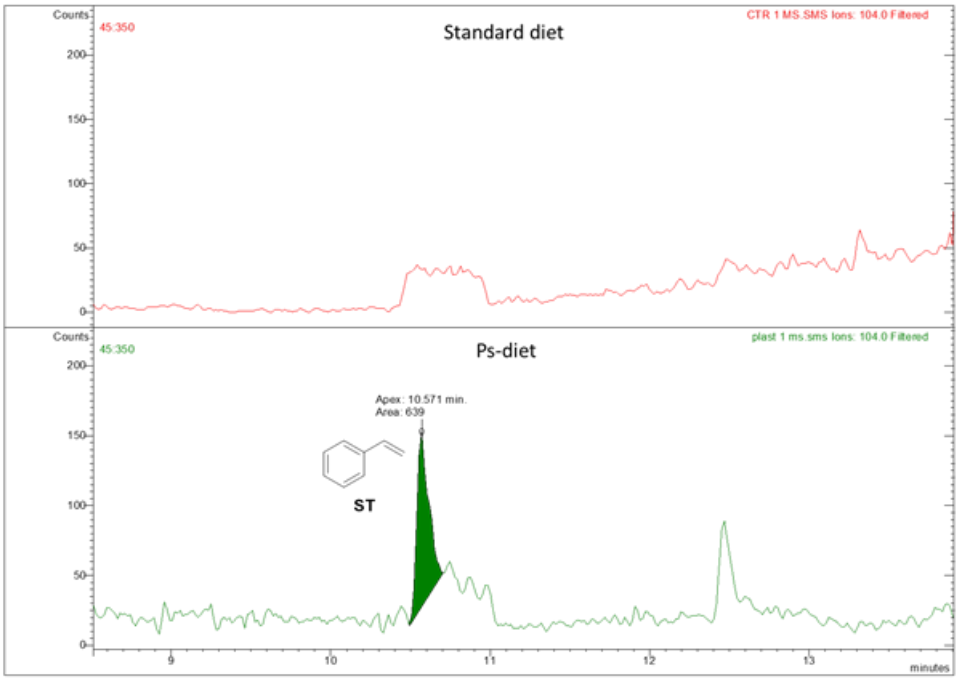
Supplemental materials

a) Chromatogram plot 1 day of feeding



b) Chromatogram plot 3 days of feeding

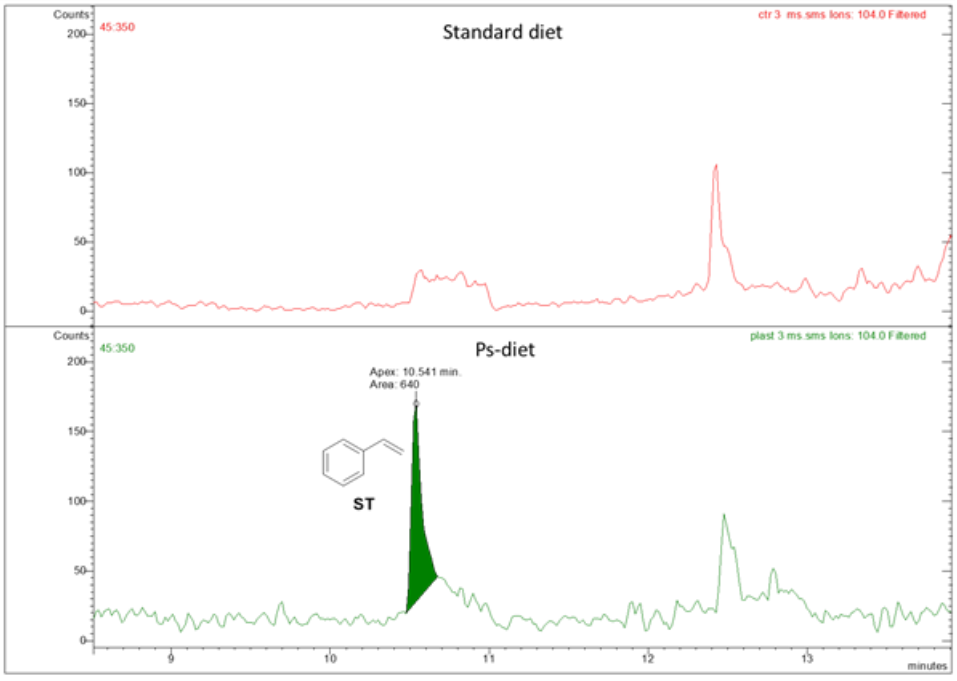


Figure S2 a, b. Chromatogram plot extracted with Gas Chromatography-Mass Spectrometry of the lyophilised intestines of Black Soldier Fly larvae, after 1 day (a) and 3 days (b) of feeding. The red spectrum represents the control (standard diet), and the green spectrum represents the treated (polystyrene diet).

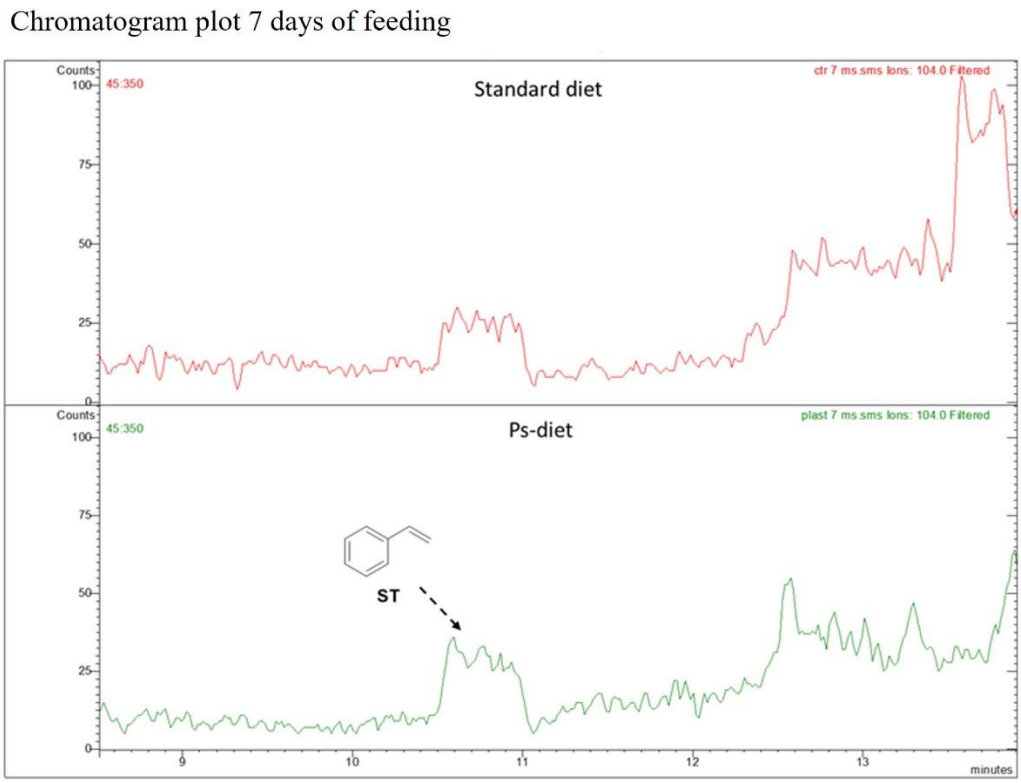


Figure S2. Chromatogram plot extracted with Gas Chromatography-Mass Spectrometry of the lyophilised intestines of Black Soldier Fly larvae, after 7 days of PS feeding. The red spectrum represents the control (standard diet), and the green spectrum represents the treated (polystyrene diet).

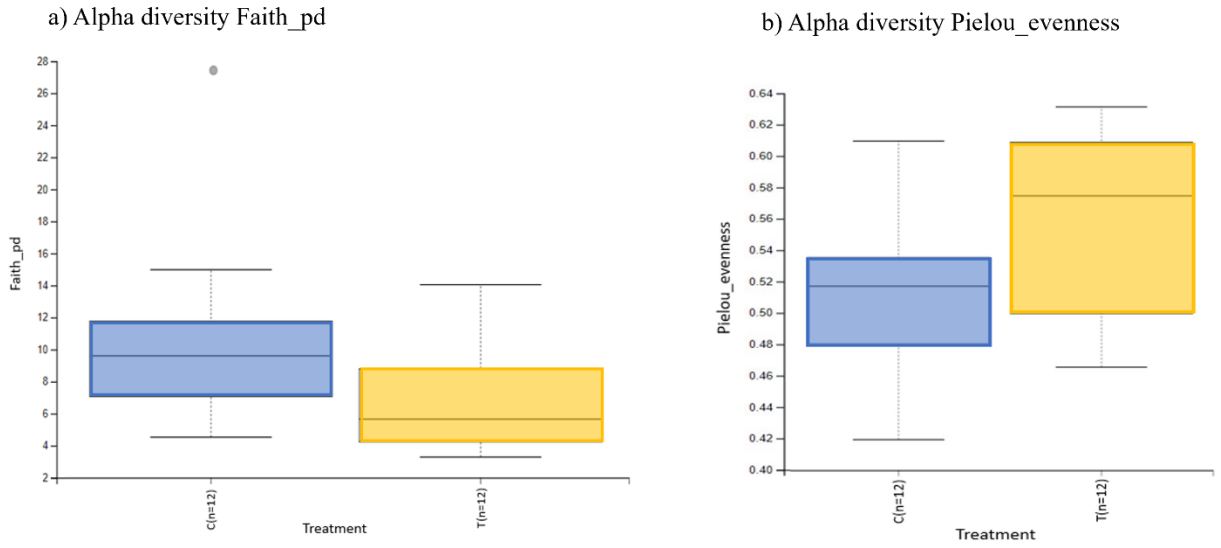


Figure S3 a, b. The Kruskal-Wallis significance boxplot for alpha diversity metrics is based on the treatment factor (control diet and treated diet). The boxplots indicate no difference in alpha diversity between the bacteria in the two treatments ($p > 0.05$). a) alpha diversity based on Faith-pd, b) alpha diversity based on Pielou-evenness. For both diversity 12 samples per treatment were analysed.