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Research Paper

Microplastics in the diet of *Hermetia illucens*: Implications for development and midgut bacterial and fungal microbiota

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ABSTRACT

In a world with a population exceeding 8 billion people and continuing to grow, pollution from food and plastic waste is causing long-term issues in ecosystems. Potential solutions may be found by exploiting insect-based bioconversion. In this context, we investigated the impact of polyvinyl chloride microparticles (PVC-MPs) on the development of *Hermetia illucens* (black soldier fly; BSF) and its midgut bacterial and fungal microbiota.

The impact of PVC-MPs was evaluated feeding BSF larvae with a PVC-MPs-supplemented diet. The larvae exposed to different PVC-MPs concentrations (2.5%, 5%, 10% and 20% w/w) developed into adults with no significant increase in pupal mortality. Faster development and smaller pupae were observed when 20% PVC-MPs was provided. The BSF larvae ingest PVC-MPs, resulting in a reduction in MPs size. Larvae exposed to PVC-MPs did not exhibit differences in gut morphology. Regarding the impact of PVC-MPs on the structure of both bacterial and fungal communities, the overall alpha- and beta-diversity did not exhibit significant changes. However, the presence of PVC-MPs significantly affected the relative abundances of Enterobacteriaceae and Paenibacillaceae among the bacteria and of Dipodascaceae and Plectospharellaceae among the fungi (including yeast and filamentous life forms), suggesting that PVC-MP contamination has a taxa-dependent impact.

These results indicate that BSF larvae can tolerate PVC-MPs in their diet, supporting the potential use of these insects in organic waste management, even in the presence of high levels of PVC-MP contamination.

1. Introduction

Plastic pollution is one of the main environmental issues, and the production of different plastic polymers has been continuously increasing over the last 50 years (Rhodes, 2018). The production, often single-use consumption, and disposal of plastics have directly led to a dramatic increase in the global amount of plastic waste (MacLeod et al., 2021). In some countries, such as the United States, European countries, India and China, municipal solid waste comprises approximately 44 % of food, leaf and vegetable wastes, 17 % of paper and cardboard and 12 % of plastic (Shiddiq et al., 2023). Organic fractions comprise a greater percentage of the municipal solid waste, with higher levels in low-income countries (where organic waste typically accounts for 50 % to 70 % of waste) than in high-income countries (where organics account for 20 % to 40 % of waste). The plastic levels generally appear high (8 % to 12 %) and they are not largely dependent on the income level of countries (United Nations Environment Programme, & International Waste Management Association, 2015). Some authors predicted that, without increased efforts to reduce plastic waste, the total amount destined for landfills or deposition in natural environments by the end of 2050 could reach approximately 12,000 Mt (Geyer et al., 2017; Sanchez-Hernandez, 2021). Once discarded, plastic waste undergoes degradation into smaller pieces, referred to as microplastics (MPs = 1–1,000 μm) and nanoplastics (NPs = 1–1,000 nm), depending on the size (Bermúdez and

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Swarzenski, 2021). Both forms are the primary source of plastic pollution (Wu et al., 2017). Food waste contains MPs at up to 91 particles/g refuse in less controlled landfills (Romano and Fischer, 2021), and there is significant evidence that both MPs and NPs have negative impacts on human and animal health (Campanale et al., 2020; Jiang et al., 2020; Stapleton, 2021).

Due to its long lifespan and good mechanical, electrical, chemical and thermal resistance properties, polyvinyl chloride (PVC) is among the most popular plastic polymers and widely used in numerous applications, including food packaging and household products (Miliute-Plepiene et al., 2021). It is one of the four major plastic components commonly found in municipal waste streams, together with polyethylene (PE), polyethylene terephthalate (PET) and polypropylene (PP) (Judi et al., 2019). These polymers contaminate organic wastes, collectively constituting more than 10 % v/v of the total mass (Judi et al., 2019). Although PVC is used at lower amounts than PET, it contains far more additives and therefore has a higher potential to induce toxicity (Marcilla et al., 2008). In particular, if unmanaged PVC wastes are subjected to open burning, this can result in the emission of toxins such as dioxins and furans (UN Global Waste Management Outlook, 2015).

Currently, municipal waste, including the organic fraction, is treated using mechanical–biological methods, such as the preliminary separation of the organic fraction from plastic and other inorganics (e.g., paper, glass, metal, cardboard). Once separated, the organic fraction may be landfilled, burned, dumped, composted or subjected to vermicomposting, among others. However, this separation process is often not complete, resulting in variable amounts of residual MPs that can contribute to contaminate the organic fraction of municipal solid waste (Judy et al., 2019; Edo et al., 2022). Generally, MPs can be abundant in solid organic waste, such as sewage sludge, livestock manure, domestic waste and human faeces (Zhou et al., 2023). The authors of a recent study estimated an amount of 12.3 particles/g in organic solid waste in a landfill nearby the Persian Gulf (Mohammadi et al., 2023). The application of solid organic waste and its biological treatment products (compost, vermicompost and biogas residues) can return nutrients and trace elements to soils; however, it can also lead to the accumulation of MPs after long-term application, aggravating soil MP contamination (Zhou et al., 2023).

In the last decades, due to urban development and population growth, adequate waste management policies have been established to promote the efficient separate collection of the organic fraction of municipal solid waste. This procedure, although it has widely become mandatory, poses significant challenges for governments worldwide. Due to its ability to convert organic waste into valuable products for energy, food, feed and agricultural applications (Newton et al., 2005; Li et al., 2011a,b; Wang and Shelomi, 2017; Schmitt and de Vries, 2020; Mohan et al., 2023), the black soldier fly (BSF) *Hermetia illucens* L. (Diptera, Stratiomyidae) has been studied for its pivotal role in waste disposal, processing and bioconversion (Nguyen et al., 2015; Fuso et al., 2021; Barrett et al., 2022). In this context, it is necessary to investigate whether the presence of plastics in the organic fraction compromises the vitality and development of BSF larvae, providing evidence of their possible application in organic waste management despite MPs contamination.

Although the BSF has been studied as part of the circular economy since the 2000 s (Kee et al., 2023), little is known about the impact of plastic particles in the substrate on both adult survival and larval development. Despite the fact that BSF larvae can grow on a wide variety of agri-food wastes, their feeding on different types of substrates can have an impact on their growth, physiology and nutritional composition (Diener et al., 2009; Nguyen et al., 2013; Banks et al., 2014; Tschirner and Simon, 2015; Koeleman et al., 2016, Spranghers et al., 2017; Cho et al., 2020; Bonelli et al., 2020; Scala et al., 2020).

Among the studies investigating the effects of various compounds on the development of BSF larvae, the impact of MPs has been largely investigated in recent years, with emphasis on the effects of some plastic polymers (PP, PE, PVC, polyamide PA and polystyrene EPS) on BSF larvae physiology and development (Cho et al., 2020; Romano and Fisher, 2021; Lievens et al., 2022, 2023; Heussler et al., 2023; Xu et al., 2023). However, the results are contradictory, ranging from no effects when using 1 % w/w PVC-MPs in the form of flat fragments with an irregular flake shape (Lievens et al., 2022) or PA-MPs at a concentration of 0.22 % w/w (Heussler et al., 2023) to delayed development with 0.22 % w/w PP-MPs (Romano and Fisher, 2021) or a lower survival rate in larvae treated with 5 % PS-MPs (Cho et al., 2020). Some authors investigated the fate of MPs within BSF larvae by describing the ingestion and excretion dynamics of fluorescent-labelled particles, excluding bioaccumulation and digestion as potential processes (Lievens et al., 2023; Heussler et al., 2023; Xu et al., 2023).

However, there are few data on the impact of MPs on the structure and functionality of the midgut microbiota and gut ultrastructure. The study of Xu et al. (2023) reported the emergence of antibiotic resistance in bacterial communities colonising the gut of BSF larvae fed with livestock faeces contaminated with PVC MPs. Similarly, De Filippis et al. (2023) focused on the bacterial community and reported the presence of genes with plastic-degrading potential in the BSF larvae gut microbiome when reared on PE and PS plastics. The gut microbiota is a key component of metazoans, including insects (Fraune and Bosch, 2010; Storelli et al., 2011; Morimoto et al., 2019), and in the absence of symbiotic microorganisms, insects can show reduced growth rates and high mortality (Ridley et al., 2012; Salem et al., 2013). In recent years, both the diversity and function of the intestinal microbiota of BSF larvae in relation to different substrates for larval rearing have been studied, demonstrating the establishment of a core bacterial community with an astonishing plasticity and explaining the dietary flexibility of these insects (Cifuentes et al., 2020; Klammsteiner et al., 2020; Schreven et al. 2021; Gold et al., 2021; Tanga et al., 2021; Chen et al., 2023, De Filippis et al., 2023).

However, the current literature does not provide a comprehensive overview of the impact of MPs in the BSF larval diet. The use of plastic materials with varying sizes, shapes and concentrations makes it difficult to compare the results of the different studies published so far, and numerous fundamental issues still need to be clarified (Lievens et al., 2023; Heussler et al., 2023), such as the ingestion, processing and secretion of different types of MPs (Lievens et al., 2022; Heussler et al., 2023) and the potential for plastic biodegradation by BSF larvae (Sanchez-Hernandez, 2021).

In this study, we focus on one type of plastic (irregular PVC spheres, approximately 150–190 μm in diameter) and assess its impacts when used in different concentrations (2.5 %, 5 %, 10 % and 20 % w/w) in the diet of BSF larvae on their gut microbiota. We consider aspects of insect postembryonic development (duration of the larval and pupal development, pupal mortality, pupal dimensions) and midgut-related parameters (midgut ultrastructure, bacterial and fungal microbiota) by comparing larvae reared with different levels of PVC-MPs contamination to control larvae without PVC-MPs in the diet. We also investigate the ingestion and size reduction of plastic fragments by the larvae, using electron microscopy.

2. Materials and methods

2.1. Origin of microplastics and electron microscopy materials

Commercial polyvinyl chloride (PVC low mol. weight – Cod. 81388, 100 % average purity) was supplied by Aldrich Chemistry (St. Louis, MO, USA). Due to the declared average powder size (approximately 150–190 μm), confirmed by scanning electron microscopy observations, the PVC particles can be classified as MPs, according to Bermùdez and Swarzenski (2021). All chemicals used for electron microscopy were supplied by Electron Microscopy Sciences (Hatfield, PA, USA), except the Epon-Araldite resin mixture, which was purchased from Aldrich

Chemistry (St. Louis, MO, USA).

2.2. Insect rearing

The BSF larvae used in this study were obtained from a colony established in 2020 at the University of Perugia (Perugia, Italy), starting from pupae bought from BugsLife Srls (Bevagna, Italy). In the colony, male and female BSF adults were kept in the same wooden and acrylic glass cage (50 x 50 x 50 cm) for mating under LED lighting (STASUN, 9,000 lm, 100 W, 5,000 Kelvin) within a controlled environment chamber (14-h photoperiod, a temperature of 28 ± 3 °C and a relative humidity maintained at 60 ± 10 %) and were provided with water and crystallised sucrose. The attractant for oviposition was Gainesville diet (50 % wheat bran, 30 % alfalfa meal and 20 % corn by weight) diluted in water (250 mL of water for 100 g of diet), offered in cylindrical pots (5 cm in diameter, 8 cm in height). The lid of the pots was perforated, and cardboard squares were inserted to provide oviposition sites for the females. These pots were checked daily, and cardboard pieces with freshly laid eggs were removed and placed in other closed pots containing wet paper to maintain the moisture content required for hatching. After 3 days, the neonate first instar larvae were transferred, using a small brush, to transparent plastic containers (16 x 14 x 5 cm) with a mesh-covered rectangular hole (10 x 5 cm) in the lids, containing the Gainesville diet mixed with water (125 mL of water and 100 g of diet) for larval development. Approximately 50–100 larvae were located in each plastic container. The pupae were moved to the above-described wooden cages (50 × 50 × 50 cm) for adult emergence. Considering that BSF larvae development occurs in six instars, characterised by variable sizes, larvae with lengths approximately 10–15 mm, belonging to the fourth or fifth instar (Barros et al., 2019), were selected to investigate the midgut-related parameters (ultrastructure, bacterial and fungal microbiota) as well as the PVC-MPs ingestion by the larvae.

2.3. Larval exposure to PVC-supplemented diet

To assess the impact of a diet enriched with PVC-MPs, BSF larvae were reared using the method described above, but they were provided with a Gainesville diet supplemented with different amounts of PVC-MPs (concentrations of 2.5 %, 5 %, 10 %, 20 % w/w) for the treatments and without PVC-MPs for the control. For each replicate, 80 g of diet in 125 mL of water was placed in the same type of plastic containers used for larval rearing. In the treatment replicates, the adequate amount of PVC-MPs was added to the diet by mixing with a spoon. To reduce individual variability, larvae from the same egg clutch were used for one treatment (50 neonate larvae) and the corresponding control replicate (50 neonate larvae). To ensure optimal growth, on days 6 and 12 from the start of the feeding experiments, 30 g of diet (with or without PVC-MPs) + 50 mL of water were added.

2.4. Insect postembryonic development in the presence and absence of PVC-supplemented diet

To evaluate the duration of the larval and pupal stages, the post-embryonic development of treated (PVC-MPs-supplemented diet) and untreated (control) insects was checked daily, recording the reaching of the pupal stage and adult emergence.

Fifty larvae were used in each replicate both for test and for control. A sample of insects from each replicate was used for evaluation of larval and pupal development duration, pupal mortality, and pupal morphometric parameters. The number of replicates for each PVC-MPs concentration (and the corresponding controls) was variable, based on insect viability: 2.5 % PVC-MPs: n = 8 replicates with a total of 257 insects sampled for the control and 241 for the treatment; 5 % PVC-MPs: n = 12 replicates with a total of 380 insects sampled for the control and 403 for the treatment; 10 % PVC-MPs: n = 10 replicates with a total of 280 insects sampled for the control and 298 for the treatment; 20 % PVC-MPs: n = 14 replicates with a total of 383 insects sampled for the control and 421 for the treatment. The pupae were measured (width and length using the stereomicroscope and weight using the precision scale), each pupa was placed in a plastic pot with a net lid. Adult sex was observed upon emergence and recorded.

2.5. Scanning electron microscopy (SEM) and energy-dispersive x-ray microanalysis (EDX)

To characterise the PVC-MPs used in the experiments, MPs were mounted on aluminium stubs using double-sided carbon tape and metallised with a thin layer of chromium (8 nm). Fifteen 10–15-mm larvae fed with a Gainesville diet supplemented with 20 % PVC-MPs, along with 15 10–15-mm larvae from the correspondent control replicates, were collected, anaesthetised with CO_2 and dissected in sodium cacodylate buffer pH 7.2 to obtain the guts. These larval guts were dissected and fixed for 3 h in a 2.5 % glutaraldehyde solution in sodium cacodylate buffer pH 7.2, repeatedly rinsed in sodium cacodylate buffer and dehydrated using ascending ethanol concentrations. Finally, the dehydrated guts were dried in an oven at 30 °C for 24 h, mounted on aluminium stubs using double-sided carbon tape, cut longitudinally with a razor blade to expose the internal contents and metallised with a thin layer of chromium (8 nm). Ten grams of Gainesville diet supplemented with 20 % PVC-MPs not exposed to larvae, and the same amount of frass collected after the completion of the larval development on the same diet, were frozen at −18 °C for 10 min, oven-dried at 40 °C for 72 h, mounted on aluminium stubs using double-sided carbon tape and metallised with a thin layer of chromium (8 nm). The samples were analysed with a field emission scanning electron microscopy (FE SEM LEO 1525, ZEISS, Oberkochen, Germany) using backscattered electrons at 15 kV and secondary electrons at 5 kV. Backscattered SEM images revealed compositional differences through contrast (brighter areas indicate the presence of material with higher atomic numbers).

The presence of chlorine (Cl) in the diet supplemented with 20 % PVC-MPs, as well as in the gut content of BSF larvae reared on it, was determined using a field emission SEM equipped with an energy-dispersive x-ray detector (EDX) (Bruker Quantax, Billerica, MA, USA). The substrate remaining after the completion of the development of the BSF larvae, called frass, was analysed using the same method. Because of its presence in PVC, Cl was used to identify PVC-MPs. In our experiments, the presence of Cl due to other materials, such as diet, containers, equipment, was null or negligible. The following parameters were applied: measurement time 5 min, accelerating voltage 15.00 kV, working distance 10.3 mm. The size of PVC-MPs in the diet and the frass was assessed based on SEM images and by measuring the coloured area (red) of each PVC-MP from digital images obtained with the EDX, using ImageJ (Schneider et al., 2012).

2.6. Transmission electron microscopy (TEM)

Five 10–15-mm larvae were anaesthetised and dissected for each rearing condition (control, 2.5 %, 5 %, 10 % and 20 % PVC-MPs) to obtain the midgut. This section of the gut was selected because it represents the region involved in the production and secretion of digestive enzymes, components of the peritrophic matrix and nutrient absorption; additionally, it is the longest part of the digestive system (Bonelli et al., 2019). The midguts were dissected and fixed for 3 h in 2.5 % glutaraldehyde in sodium cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA, USA) with a pH of 7.2. Subsequently, they were rinsed repeatedly in sodium cacodylate buffer and post-fixed for 1 h at 4 °C in 1 % osmium tetroxide in sodium cacodylate buffer. The samples were then repeatedly washed in the same buffer, dehydrated by using ascending ethanol concentrations and finally embedded in an Epon–Araldite resin mixture. Afterwards, ultra-thin sections were cut using a Leica EM UC6 ultramicrotome (Leica Microsystem GmbH, Wetzlar, Germany), collected on copper grids coated with Formvar (Sigma-Aldrich, St Louis,

MO, USA), stained with uranyl acetate and lead citrate and examined with a Philips EM 208 TEM (Philips, Eindhoven, the Netherlands).

2.7. Gut tissue collection for microbiological analysis

Five 10–15-mm larvae from three replicates for each PVC-MPs concentration (2.5 %, 5 %, 10 % and 20 % PVC) and five 10–15-mm larvae for each of the corresponding controls were anaesthetised by CO_2 and dissected to obtain the midgut for microbiological analysis, as reported elsewhere (Bruno et al., 2019). Larvae were washed with 70 % ethanol in autoclaved distilled water and then dissected under sterile conditions using a stereomicroscope. Sterile tweezers and scissors were used to prevent cross-contamination of the samples. Each gut was dissected and isolated in autoclaved sodium cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA, USA) pH 7.2 in a sterile Petri dish (9 × 1.3 cm). The dissected gut tissues were cleansed to remove fat, washed with pure autoclaved water, and the anterior and posterior sections of the gut were removed by scissors. For the dissection of the larvae from each replicate a new Petri dish was used, and tweezers and scissors were cleaned with 70 % ethanol in pure autoclaved water. Immediately after dissection, the five midguts of each replicate were transferred to a sterile 2-mL reaction tube. Before DNA extraction, the weight of collective midgut samples was determined.

2.8. Total DNA extraction and quantification

The DNA was extracted using 0.25 g of larval midgut for each sample using the Quick-Start DNeasy® PowerSoil® Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The DNA concentration was quantified using the QuBit 3.0 Fluorometer Assay (Life Technologies Corporation, Carlsbad, CA, Stati Uniti).

2.9. Bacterial and fungal community quantification

Quantitative polymerase chain reaction (qPCR), targeting the bacterial 16S rRNA gene and the fungal ITS region, was performed following the procedure described in Sannino et al. (2022). In brief, to quantify the microbial abundance of the control and treatment samples for each PVC concentration, the primers Eub338 (5′-ACTCCTACGGGAGGCAGCAG-3′) and Eub518 (5′-ATTACCGCGGCTGCTGG-3′) for bacteria and NS91 (5′-GTCCCTGCCCTTTGTACACAC-3′) and ITS51 (5′-ACCTTGTTACGACTT TTACTTCCT C-3′) for fungi were used (Fierer et al., 2005; Onofri et al., 2012). Fungal and bacterial abundances were expressed as the number of copies of rDNA genes per nanogram of DNA on a log scale.

2.10. Bacterial and fungal DNA amplification and metabarcoding sequencing

Bacterial DNA amplification was carried out targeting the V3 and V4 hypervariable regions of microbial 16S rDNA genes, using the primers Pro341F (5′-CCTACGGGNBGCASCAG-3′) and Pro805R (5′-GACTACNVGGGTATCTAATCC-3′) (Takahashi et al., 2014). Fungal DNA amplification was carried out targeting the ITS2 region, using the primers IlluAdp_ITS3_NeXTf (5′-CATCGATGAAGAACGCAG-3′) and IlluAdp_ITS4_NeXTr (5′-TCCTCCGCTTATTGATATGC-3′) (Tedersoo et al., 2015). Metabarcoding sequencing was performed on the Illumina MiSeq platform (2 x 300-bp reads) by BMR Genomics (Padova, Italy). Bacterial and fungal raw sequences were separately processed using AMPtk v. 1.5.1 (Palmer et al., 2018). Reads were demultiplexed and trimmed, and chimera were removed. After discarding low-quality reads, reads < 250 bp, singletons and rare OTUs (<5 reads in the whole dataset), reads were clustered to identify molecular operational taxonomic units (OTUs) with a 97 % identity threshold, using the VSEARCH (v. 2.3.2) algorithm (Rognes et al., 2016). Finally, taxonomy was assigned by using the hybrid SINTAX/UTAX database (Edgar, 2010). Bacterial taxonomy was updated following the List of Prokaryotic names with Standing in Nomenclature (LPSN) (https://www.bacterio.net). Sequences were archived in the NCBI SRA database linked to BioProject accession numbers PRJNA999508 for bacteria and PRJNA999505 for fungi.

2.11. Data analysis and statistics

The duration of the larval and pupal stages, as well as the pupal morphometric parameters (width, weight and length), in treated and control replicates were compared for each PVC-MPs concentration using a one-way repeated measures analysis of variance (ANOVA), considering the sex as a factor. Pupal mortality in controls and PVC-treated replicates was compared with the Student *t*-test for dependent samples. The sizes of PVC-MPs in the diet and in the frass were compared using the Student *t*-test for independent samples. Prior to the analysis, the data were subjected to the normality test (Shapiro-Wilk) and the equal variance test and, when necessary, to a Box-Cox transformation to reduce heteroscedasticity (Sokal and Rohlf, 1998).

Statistical analyses regarding the bacterial and fungal microbiota were performed using statistical multi-packages of the R software version 4.0.3 (R core Team 2020). Bar plots showing the most abundant bacterial and fungal families were generated using the ggplot 2 package (Wickham, 2016). Microbial alpha-diversity (Richness and Shannon-H indices) were calculated via phyloseq in R (McMurdie and Holmes, 2013). Differences in bacterial and fungal alpha-diversity were tested via ANOVA, followed by a Tukey's pairwise post hoc test in R, using the ggpubr function of the ggplot2 package (Wickham, 2016). Microbial beta-diversity was calculated by the Bray–Curtis distance, and differences were tested using the permutational multivariate analysis of variance (PERMANOVA) by the function 'adonis', implemented in the vegan package in R (Oksanen et al., 2017). Generalized linear models (GLMs) were used to analyse bacterial and fungal data by the Mass package (Venables and Ripley, 2002). Sequences belonging to bacterial and fungal taxa (proportion of the total amounts) were expressed as the log odds ratio of PVC-treated *vs* control samples. A log odds ratio > 0 indicated a higher microbial parameter found in the PVC-treated samples compared to the control, a value = 0 indicated the same value, and a value < 0 indicated a lower value in the PVC-treated samples compared to the control (Barili et al., 2023). For all models, the PVC concentration (2.5 %, 5 %, 10 % and 20 %) was included as an independent factor variable. Co-occurrences among microbiological parameters (bacterial and fungal families with a relative abundance > 1 %) were calculated based on the Pearson correlation coefficient separately for control and treated samples, using the corrplot package in R (Wei and Simko, 2021).

3. Results

3.1. Impact of PVC-MPs on insect postembryonic development

The BSF larvae exposed to different concentrations of PVC-MPs in the diet at 28 °C regularly developed into adults, with a variable timeframe of larval development ranging from 25 to 45 days. Concentrations of PVC-MPs higher than 2.5 % resulted in a significantly ($P < 0.05$) faster larval development (Fig. 1a, Table S1). Pupal mortality rate was approximately 10 %–20 %, with no statistically significant differences ($P < 0.05$) compared to the control group (Fig. 1b, Table S1). The duration of the pupal stage ranged between 8 and 14 days (Fig. 1c), with a significantly ($P < 0.05$) faster development only with 20 % PVC-MPs contamination (Fig. 1c, Table S1). The same PVC-MPs concentration also induced a significant ($P < 0.05$) decrease in all morphometric pupae parameters (weight, length and width) (Fig. 1d, S1, Table S1).

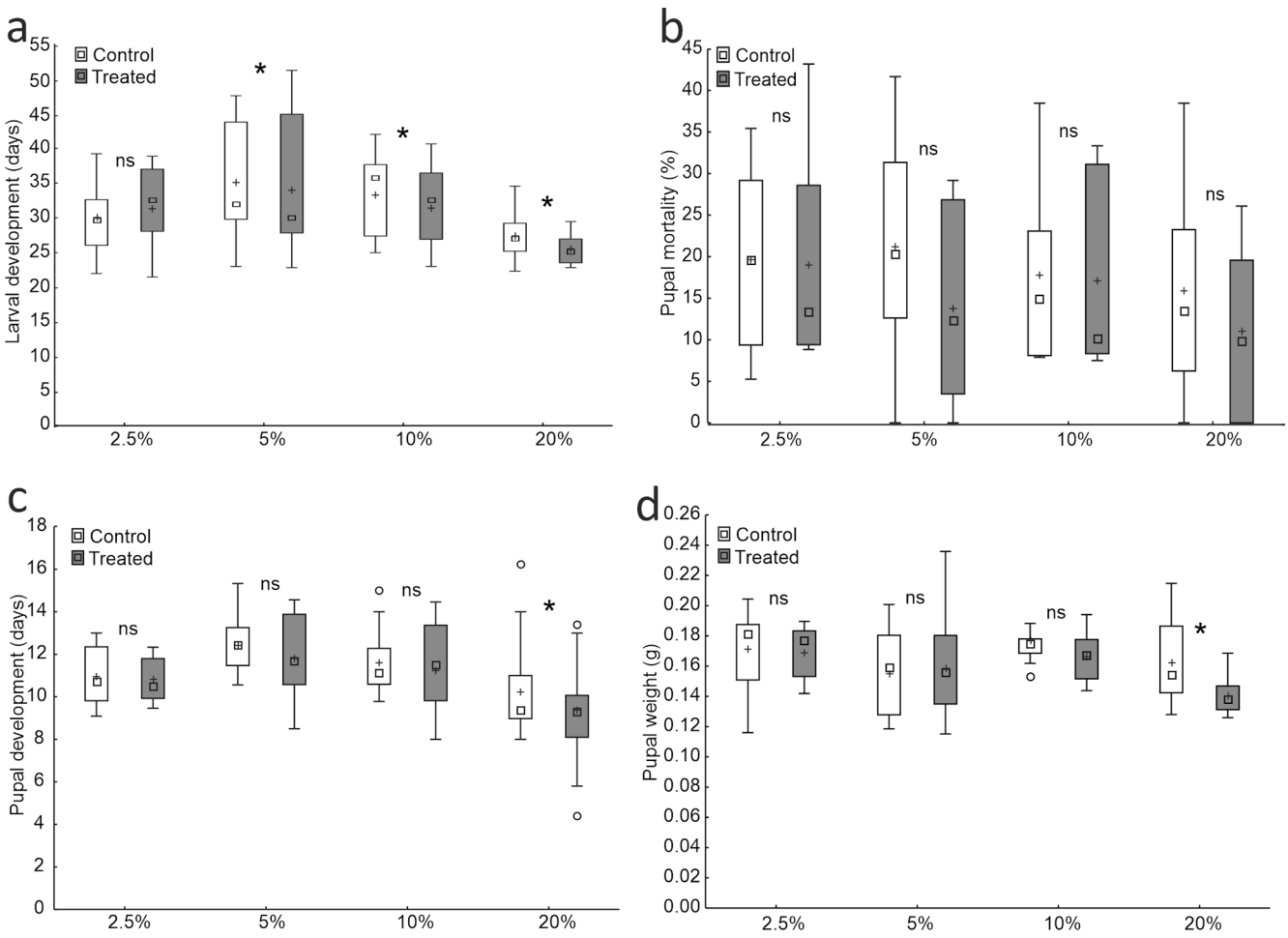

Fig. 1. Impact of diet enriched with PVC-MPs (2.5, 5, 10 and 20 %) on BSF larvae and pupae development a) Larval development duration (days); b) Pupal mortality (%); c) Pupal development duration (days); d) Pupal weight (g). Boxplots show the interquartile range and the medians, whiskers indicate the 1.5 × interquartile range, "○" shows outliers and + shows the mean. Treated and control for each PVC concentration are compared; boxplots with * significantly differ at $P < 0.05$ (for a: *t* test for dependent samples, for b, c, d: one-way repeated measures ANOVA) (see Table S1).

3.2. *Pvc-mps in the larval midgut*

The PVC-MPs used in the experiments, as observed via SEM, appeared as irregular spheres with globules on their surface. Most of the particles had a size between 15,000 and 20,000 μm^2 (20,273 ± 911 μm^2 (mean ± SE)) (Fig. 2a, Fig. S2). Their presence in the midgut, where it was not possible to determine the MPs size, (Fig. 2b, d), suggests that these particles are easily ingested with the diet and are not rejected by the insect. In the SEM images, PVC-MPs were visible as brighter areas (arrows in Fig. 2b) due to the presence of elements with higher atomic numbers compared to the darker areas. The presence of chlorine (Cl) in the PVC-MPs was highlighted in red, using SEM equipped with energy-dispersive X-ray microanalysis (Fig. 2d, f, h). No chlorine was observed in the midgut of larvae reared with the (PVC-free) control diet (Fig. 2c). The size (area μm^2) of PVC-MPs found in the larval frass (9,646 ± 1,289 μm^2 (mean ± SE)) (Fig. 2g, h) was significantly reduced compared to the size of non-ingested PVC-MPs (14,314 ± 1,453 μm^2 (mean ± SE)) (Fig. 2e, f, Fig. S3) ($t = 2.27$, d.f. = 52, $p = 0.027$), as visible via SEM and EDX microanalysis.

The larvae exposed to different concentrations of PVC-MPs did not show detectable effects on the fine midgut morphology (Fig. 3). The anterior portion of the midgut exhibited a thick epithelium with electron-dense vesicles in the apical part of the columnar cells, under the microvilli, both in the control and treated larvae (Fig. 3a, b). The medium (Fig. 3c, d) and posterior (Fig. 3e, f) portions of the midgut showed extremely elongated microvilli, slightly shorter in the presence of PVC-MPs (Fig. 3d, f) than in the control (Fig. 3c, e), particularly in the posterior portion (Fig. 3e, f), although always well developed. The peritrophic matrix was continuous and visible with a regular structure, also in the treated insects reared on a diet with 20 % PVC-MPs (Fig. 3d).

3.3. *Impact of PVC on bacterial and fungal abundance and diversity*

After bioinformatic analyses, a total of 867,081 and 1,784,977 reads for bacteria and fungi were found, respectively. The means and standard deviations for each sample are reported in Supplementary Table S2.

From a quantitative viewpoint, the results of the qPCR indicate no significant ($P < 0.05$) impact of PVC on both bacterial and fungal rDNA gene copy numbers (Fig. S4).

Metabarcoding analysis revealed that raw sequences clustered bacterial OTUs in 6 phyla, 18 classes, 26 orders, 66 families and 130 genera. The percentage of unclassified bacteria (calculated as means among all samples) was 3 % at the phylum level, 9 % at the class level, 19 % at the order level, 20 % at the family level and 24 % at the genus level. The most abundant bacterial phyla were Bacillota (former Firmicutes, from 38.02 % to 61.19 %), Pseudomonadota (former Proteobacteria, from 11.76 % to 37.47 %) and Bacteroidota (former Bacteroidetes, from 8.95 % to 24.67 %) (Table S3). Enterobacteriaceae was the most abundant family (from 30.11 % to 32.84 %), followed by Lachnospiraceae (from 10.82 % to 20.30 %), Paenibacillaceae (from 9.91 % to 21.12 %) and Porphyromonadaceae (from 2.27 % to 14.09 %) (Fig. S5a). Finally, *Paenibacillus* (from 9.63 % to 21.01 %), *Providencia* (from 1.61 % to

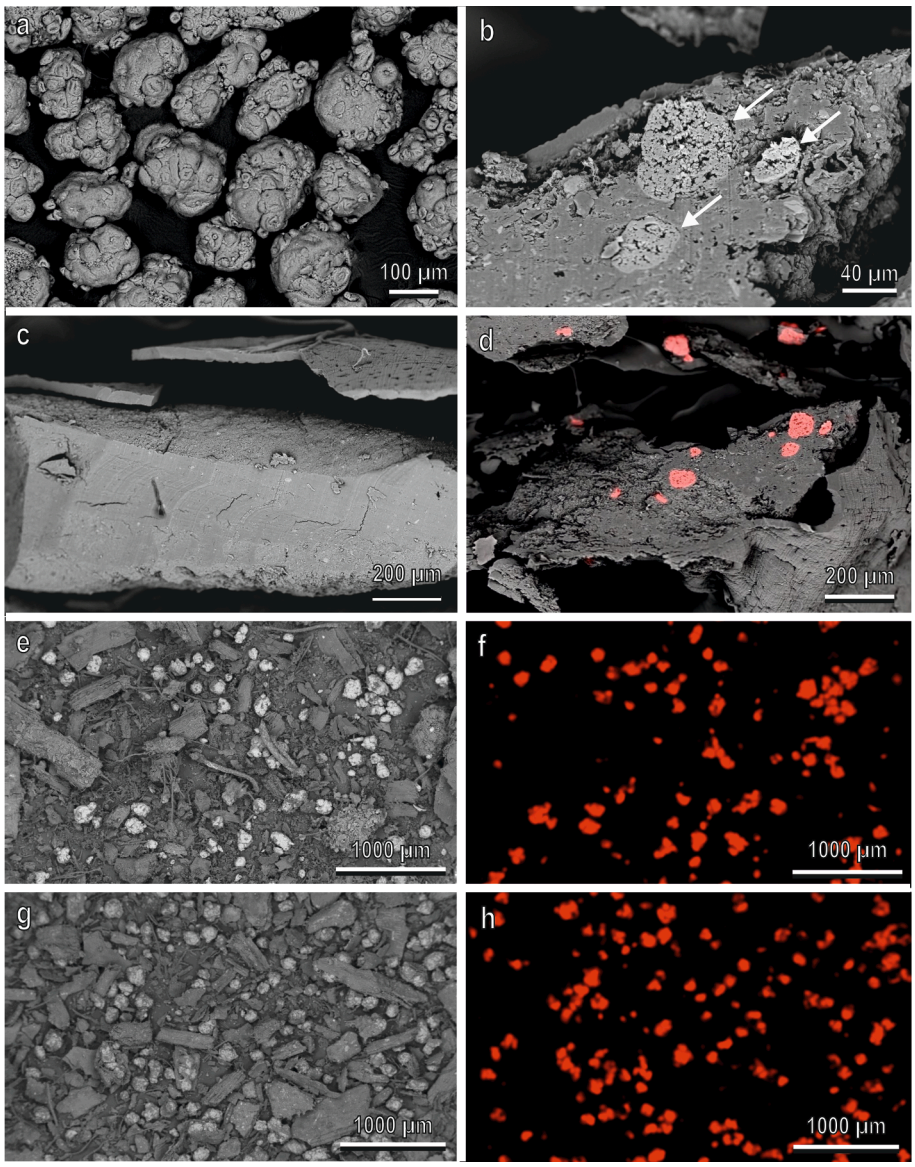

Fig. 2. SEM and EDX microanalysis of PVC-MPs a) PVC-MPs used in the experiments; b) Backscattered image of BSF larvae midgut containing PVC-MPs, visible as brighter areas (arrows) owing to the presence of higher atomic number elements (Cl) than darker ones; c-d) Longitudinal section of the midgut of a control larva (c) and a treated larva reared on a diet with 20% PVC-MPs (d); e) SEM image and f) spatial distribution of Cl in the 20% PVC diet; g) SEM image and h) spatial distribution of Cl in the 20% PVC frass derived from rearing BSF larvae on the same diet. Chlorine, indicating the PVC particles, is visible in red.

32.44 %), *Dysgonomonas* (from 2.23 % to 13.76 %), *Clostridium* (from 4.10 % to 8.20 %) and *Bacillus* (from 0.64 % to 15.77 %) were the most abundant bacterial genera (Table S3).

Fungal OTUs were clustered in 7 phyla, 13 classes, 30 orders, 33 families and 40 genera. The percentage of unclassified fungi (calculated as means among all samples) was 0 % at the phylum level, 0.05 % at the class level, 0.05 % at the order level, 0.7 % at the family level and 74 % at the genus level. The most abundant fungal phylum was Ascomycota (from 54.44 % to 99.70 %) (Table S4). Dipodascaceae (from 18.21 % to 57.21 %), Microascaceae (from 0.32 % to 21.13 %), Nectriaceae (from 0.63 % to 53.86 %) and Trichosporonaceae (from 0.05 % to 45.45 %) were the most abundant families (Fig. S5b). At the genus level, despite the strong prevalence of unclassified fungal OTUs, the consistent presence of *Acrostalagmus* (maximum value 71.10 %), *Trichosporon* (maximum value 45.45 %) and *Aureobasidium* (maximum value 8.53 %) was observed in some of the samples (Table S4).

Likewise, the analysis of the overall alpha-diversity (calculated by Richness and Shannon-H indexes) and beta-diversity did not show any significant ($P < 0.05$) differences for both bacteria and fungi as a result of PVC addition (Tables S5 and S6, respectively). However, the presence of PVC-MPs significantly ($P < 0.05$) affected the relative abundances of specific bacterial and fungal taxa, suggesting that this polymer could have a taxon-dependent impact. The bacterial family Enterobacteriaceae exhibited a significantly ($P < 0.05$) higher abundance in the midgut of larvae reared on a diet with 20 % PVC-MPs, whereas the family Paenibacillaceae showed significantly ($P < 0.05$) higher abundances in the midgut of larvae reared on a diet with 2.5 % and 20 % PVC-MPs (Fig. 4a). Among the fungi, the presence of 20 % PVC-MPs in the diet significantly ($P < 0.05$) decreased the abundance of Dipodascaceae (Fig. 4b). A fluctuating trend was observed for Plectosphaerellaceae, which exhibited a significantly ($P < 0.05$) lower abundance in the midgut of larvae reared on a diet with 5 % PVC-MPs and showed the opposite trend in the presence of PVC-MP concentrations higher than 10 % (Fig. 4b).

The correlations among the most abundant (relative abundance > 1 %) bacterial and fungal families found in the midgut of larvae reared on

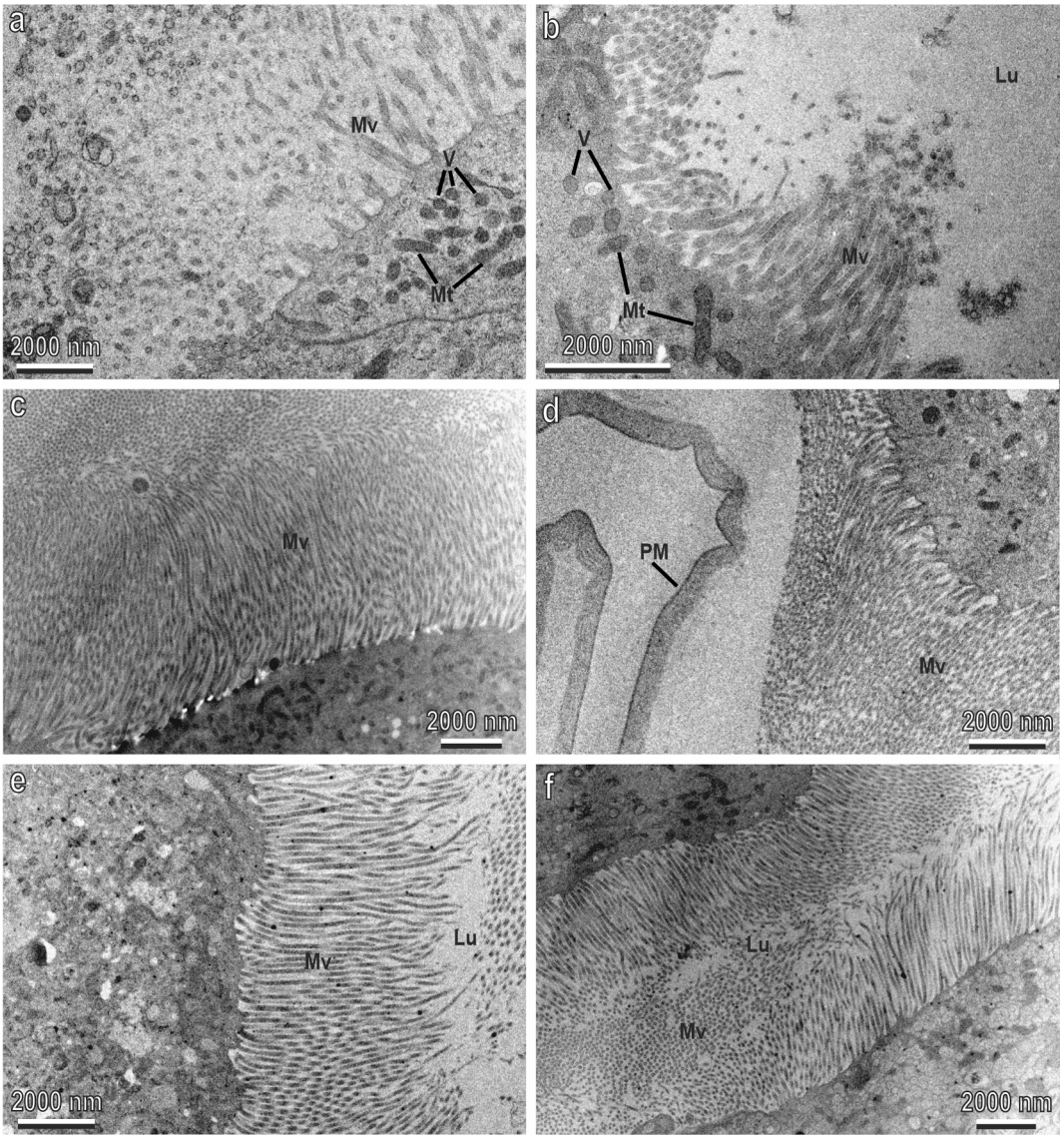

Fig. 3. Cross section of the midgut of BSF larvae reared in control (a,c,e) and treated conditions (20% PVC-MPs) (b,d,f), observed by TEM. a-b) anterior portion of the midgut; c-d) medial portion of the midgut; e-f) posterior portion of the midgut. Mv, microvilli; Lu, lumen; Mt, microtubules; V, vesicles; PM, peritrophic matrix.

a PVC-supplemented diet and in the control group were calculated. Significant ($p < 0.05$) intra-bacterial correlations were found in the midgut of larvae reared on a PVC-free diet between i) Alcaligenaceae *vs* Flavobacteriaceae and Caryophanaceae (former Planococcaceae) (positive); ii) Lachnospiraceae *vs* Paenibacillaceae (positive); iii) Lachnospiraceae *vs* Alcaligenaceae, Enterococcaceae and Porphyromonadaceae (negative); iv) Porphyromonadaceae *vs* Paenibacillaceae (negative). Among the intra-fungal significant ($P < 0.05$) interactions, only a positive correlation between Aureobasidiaceae and Nectriaceae was found. Considering bacteria *vs* fungi, significant ($p < 0.05$) correlations were found between i) Trichisporonaceae *vs* Alcaligenaceae and Caryophanaceae (positive); ii) Flavobacteriaceae *vs* Plectoshaerellaceae (positive); iii) Lachnospiraceae *vs* Trichosporaceae (negative) (Fig. S6a and Table S7).

Overall, the transition from a PVC-free diet (control) to a diet supplemented with increasing concentrations of PVC resulted in some changes in the complex network of interactions among the most abundant (relative abundance > 1 %) bacterial and fungal taxa occurring in the BSF larvae midgut microbiota (Fig. S6, Tables S6 and S7). Significant ($P < 0.05$) bacteria *vs* bacteria interactions were found in the midguts of larvae reared on a PVC-supplemented diet, namely for i) Alcaligenaceae *vs* Flavobacteriaceae (positive); ii) Lachnospiraceae *vs* Alcaligenaceae and Flavobacteriaceae (negative); iii) Bacillaceae *vs* Enterobacteriaceae (negative); iv) Enterococcaceae *vs* Paenibacillaceae (negative). Significant ($P < 0.05$) bacteria *vs* fungi interactions were found for the following combinations: i) Trichosporonaceae *vs* Alcaligenaceae, Enterobacteriaceae and Flavobacteriaceae (positive); ii) Lachnospiraceae *vs* Microascaceae (positive); iii) Caryophanaceae *vs* Microascaceae (negative); iv) Plectoshaerellaceae *vs* Bacillaceae (positive). However, no significant ($P < 0.05$) positive and negative correlations were found considering fungi *vs* fungi interactions in treated samples (Fig. S6b and Table S8).

4. Discussion

Numerous invertebrate organisms (e.g., earthworms) can easily ingest and accumulate MPs (Cole et al., 2013), sometimes with some negative effects on their growth and physiology (Huerta Lwanga et al., 2016). Overall, the results reported in this study suggest that the BSF larvae were only marginally affected by the presence of PVC-MPs in their diet. Larvae reared on a diet supplemented with a concentration of PVC-MPs up to 10 % regularly developed into adults, within a variable

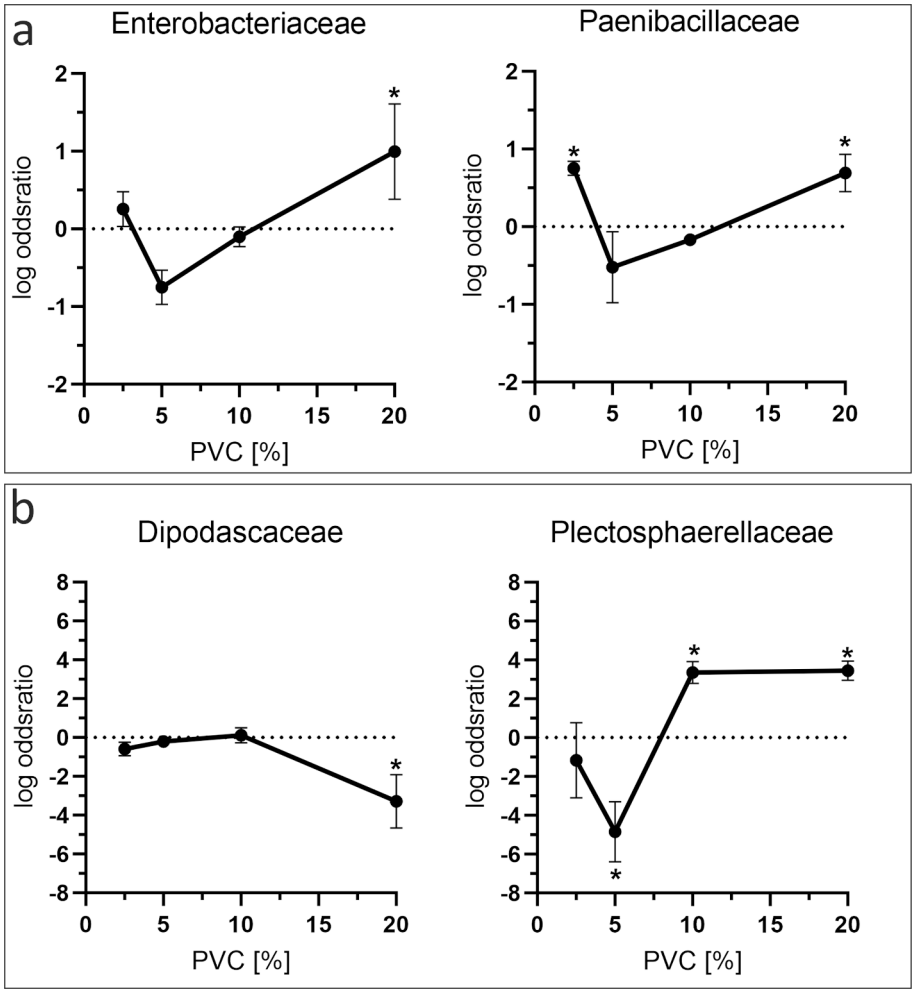

Fig. 4. Impact of PVC-MPs on the bacterial and fungal abundance in the midgut of BSF larvae. Log odds ratio of bacterial (a) and fungal (b) families with a relative abundance > 1 %. A log odds ratio > 0 indicated a higher relative abundance found in the samples treated with PVC than in the control, a value = 0 indicated the same value, while a value < 0 indicated a lower value in the PVC-treated soil than in the control. Asterisk showed significant ($P < 0.05$) differences tested by GLM.

timeframe ranging from 25 to 45 days, exhibiting similar growth patterns and becoming pupae of the same size as the corresponding controls. The development time of these larvae was longer than that of individuals reared on different diets in previous experiments, which ranged from 22 to 24 days (Tomberlin et al., 2002). This can be due to different experimental conditions and can be neglected because in our experiments the treatment and control larvae were reared exactly under the same conditions. In contrast, shorter development times and smaller pupae (width, weight and length) were obtained when the larvae were reared on a diet supplemented with 20 % PVC-MPs. This amount of plastic corresponds to an extremely high level of contamination, both considering MPs in farmlands receiving organic fertilisers (0.34 MPs/kg) or unmanaged landfills (91 MPs/ Kg) (Romano and Fisher, 2021), along with the total amount of plastic in municipal solid waste, which has been estimated to be approximately 12 % (Shiddiq et al., 2023). The control and treated replicates appear similar in terms of pupal mortality, suggesting that the negative effects on larval growth found in larvae treated with 20 % PVC-MPs were not caused by plastic toxicity. Likewise, a similar amount of polystyrene in previous studies did not affect BSF larvae survival but decreased their ability to reduce the substrate (Cho et al., 2020). The toxic effects of MPs are generally linked to gut epithelium damage, leading to inflammation and multiple responses of the immune system. Similarly, the ingestion of plastic fragments may have direct adverse effects on the insect gut epithelium, such as obstructing the digestive canal, causing abrasion and destruction of the epithelium and leading to cell lysis (Sanchez-Hernandez, 2021). None of these effects could be observed in the BSF larvae reared on a diet supplemented with 20 % PVC-MPs; instead, they showed a regular development of the gut epithelium and microvilli.

In other insects, it is assumed that the protective function of the peritrophic matrix can be compromised by MPs, for example, by breaking it due to MPs-mediated abrasion or because particles are small enough to cross the porous structure of the peritrophic matrix (Sanchez-Hernandez, 2021). In contrast, in our study, the peritrophic matrix of larvae reared on a diet supplemented with 20 % PVC-MPs was continuous and arranged in a regular shape, as a non-cellular semipermeable structure formed of proteins, glycoproteins and chitin that typically separates the midgut lumen from the epithelium in insects.

The images of the midgut content, obtained by SEM equipped with EDX microanalysis, clearly demonstrate that the BSF larvae were able to ingest PVC-MPs, in agreement with some recent studies employing fluorescent-labelled MPs (Lievens et al., 2023; Heussler et al., 2023). Lievens et al. (2023) report that MPs ingestion depends on the concentration of the polymer and the size of the larval mouth opening, which must be larger than the available particles. This supports the results obtained in our study, where mature larvae (usually characterised by a mouth opening of around 110 μm – Lievens et al., 2023) were able to ingest the PVC-MPs supplemented in their diet. Lievens et al. (2023) also reported that no significant amount of PVC was removed from the rearing substrate, with no apparent degradation or bioaccumulation of

the polymer during its passage through the midgut. This is in agreement with Heussler et al. (2023), who observed the simple ingestion and excretion of MPs without degradation or bioaccumulation in BSF larvae. Interestingly, the size of the globular PVC-MPs used in our study was significantly ($P < 0.05$) reduced during their passage through the midgut, although their shape was not apparently changed. Our PVC-MPs differed in shape and size from those used in previous investigations, where the MPs size and shape remained unchanged upon ingestion (Heussler et al., 2023; Lievens et al., 2023). It is not possible to discriminate the exact cause(s) of this size reduction, although, based on the hypotheses formulated by Lievens (2023), mechanical crushing of the particles by the mandibular-maxillary apparatus of the larvae appears to be a likely explanation. We suppose that BSF larvae are able to grind irregular PVC-MPs, in contrast to the smooth and smaller polyethylene MPs used by Lievens et al. (2023). In agreement with this hypothesis, our PVC-MPs were reduced from the initial size of 14,314 ± 1,453 μm^2 (mean ± SE) to the size of 9,646 ± 1,289 μm^2 (mean ± SE) in the larval frass, which is exactly the right size to be ingested according to Lievens et al. (2023). Some degree of degradation of PVC by bacterial and fungal communities colonizing the midgut cannot be excluded although it is difficult to assess due to the brief contact between the plastic material and the microbiota during the larval midgut passage. Considering the presence of genes involved in the degradation of plastics (e.g., PE and PS) in the BSF larvae microbiome as previously suggested by De Filippis et al. (2023), a level of degradation by microbial communities colonising the BSF larvae midgut can be considered.

The qPCR of the gut microbiota showed no significant ($P < 0.05$) impact of PVC on bacterial and fungal abundances and on the overall alpha- and beta-diversity. This suggests that the presence of the polymer in the diet of BSF larvae neither had an effect on the amount of rDNA bacterial and fungal gene copy numbers nor on the overall structure of the bacterial and fungal communities. This is consistent with previous studies reporting that the core microbiota of BSF larvae did not change significantly over the course of larval development (Cifuentes et al., 2020) or in relation to the type of diet (Klammsteiner et al., 2020). However, other taxa can vary over time. Li et al. (2022) reported that the bacterial community composition changes depending on the larval instar.

The relative abundances of the bacterial phyla found (Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria) are in line with previous findings on BSF larvae gut microbiota (Cifuentes et al., 2020; Klammsteiner et al., 2020). However, despite the apparent overall stability of the BSF larvae gut microbiota when evaluated using the classic biological diversity indices, the relative abundances of some bacterial and fungal taxa were significantly affected by the presence of PVC-MPs. Among the bacteria, the families Enterobacteriaceae and Paenibacillaceae exhibited higher abundances in larvae reared on a diet supplemented with 20 % PVC-MPs.

Recent studies have demonstrated the ability of some Coleoptera, such as *Tenebrio molitor*, *T. obscurus*, *Tribolium castaneum* and *Zophobas atratus*, and Lepidoptera, such as *Plodia interpunctella*, *Achroia grisella* and *Galleria mellonella*, to consume various non-biodegradable plastic polymers, as reviewed by Sanchez-Hernandez (2021); however, for Diptera, there are no such data. The term “plastivore”, in particular, has been introduced in this context to identify plastic-eating insect larvae able not only to chew and ingest plastics but also to degrade them via depolymerisation, with the involvement of gut microbial symbionts (Yang et al., 2015. Cassone et al., 2020; Peng et al., 2022). These studies postulate that the presence of plastic particles in the diet leads to a shift in the relative abundances of some taxa occurring in the gut microbiota, namely Enterobacteriaceae, Enterococcaceae and Streptococcaceae. This shift is presumably attributable to a possible plastic-degrading activity expressed by these bacterial taxa (e.g., *Citrobacter*) (Sanchez-Hernandez, 2021; Peng et al., 2022). Consistent with our results, Peng et al. (2020) described Enterobacteriaceae as the most abundant family of the gut microbiota of *T. molitor* larvae reared on a diet supplemented with PVC. These authors reported that the suppression of gut microbial taxa with antibiotics severely inhibited PVC degradation in *T. molitor*, suggesting that such activity is gut-colonising microbe-dependent. Regarding Paenibacillaceae, some PVC-degradation experiments have shown the contribution of the former genus *Bacillus* (now re-classified as *Brevibacillus*) in decreasing the quantity of PVC (Giacomucci et al., 2019; Anwar et al., 2016). Xu et al. (2023) observed a significant reduction in organic matter-decomposing bacteria (Pseudomonadales, Coriobacteriales, Lachnospirales and Ruminococcaceae) and an increase in pathogenic taxa such as Enterococcaceae, Hungateiclostridiaceae and Clostridia in the gut and faeces of BSF larvae reared on a PVC-enriched diet. However, similar results were not obtained in the present research.

Regarding the fungal diversity observed in our BSF larvae gut microbiota, it is only partially consistent with the current literature. Previous studies have reported that the most abundant fungal genera in the BSF larvae gut microbiota were fermentative yeasts, such as *Cyberlindnera, Candida, Kodamaea, Meyerozyma, Pichia* and *Saccharomycodes*, whereas filamentous fungi were less prevalent (Varotto Boccazzi et al., 2017; Tanga et al., 2021; Vitenberg and Opatovsky, 2022). However, the most abundant fungal genera found in the present study included both filamentous (*Acrostalagmus*), yeast (*Trichosporon*) and dimorphic (*Aureobasidium*) life forms. Among the fungal families, Dipodascaceae, previously reported as the most abundant taxon in the BSF larvae gut by Vitemberg et al. (2022), along with Aureobasidiaceae and Microascaceae, were the most abundant ones. These differences could be attributed to differences in experimental designs (e.g., the type of environment and feed, and the biodiversity herein present), which may influence the BSF larvae midgut fungal community composition (Tanga et al., 2021; Vitenberg and Opatovsky, 2022).

The high concentration of PVC significantly decreased the abundance of members of the family Dipodascaceae, whereas a fluctuating trend was observed for the family Plectosphaerellaceae. The abundance of Plectosphaerellaceae first decreased at low PVC concentrations and subsequently significantly increased when the PVC concentration exceeded 10 %. The genus *Acrostalagmus* appeared to be the responsible for the higher abundance of the family Plectosphaerellaceae. Although there is limited information about the presence and significance of this filamentous fungus in the BSF larvae gut microbiome, its cosmopolitan distribution across different ecological environments suggests its possible involvement in PVC degradation through its putative ability to express a broad spectrum of enzymatic activities (Shi et al., 2023).

The putative role of MPs as surfaces for the formation of microbial biofilms should also be considered when evaluating bacterial and fungal abundance variations in the midgut of BSF larvae reared with a PVC-supplemented diet (De Tender et al., 2017; Delacuvellerie et al., 2019). Biofilm formation can occur in the midgut of insects, considering that the microbial colonisation of MPs surfaces can require from several minutes to a few hours (Cholewińska et al., 2022). Considering that the excretion of MPs by BSF larvae and the absence of accumulation have been confirmed (Lievens et al., 2023), the formation of a biofilm on the MPs surface passing through the midgut of larvae may interfere with the diversity and magnitude of both bacterial and fungal microbiota colonising the midgut and being released in the environment as frass.

The results obtained by Pearsońs correlation analysis indicate some changes in the complex network interactions among the most abundant (relative abundance > 1 %) bacterial and fungal taxa within the BSF larvae midgut microbiota when transitioning from a PVC-free diet (control) to a diet supplemented with increasing concentrations of PVC. Although certain key families, such as Alcaligenaceae, Lachnospiraceae and Trichospororaceae, remained central nodes in both scenarios, distinct variations were observed in the presence of PVC. Enterobacteriaceae and Bacillaceae displayed both positive and negative correlations with other taxa, reaffirming their pivotal roles within the gut microbiota of larvae reared on a PVC-supplemented diet. Additionally, the fungal family Microascaceae emerged exclusively within the network of interactions observed when PVC was introduced. Members of

this family encompass saprobic and plant pathogenic species, with some even exhibiting opportunistic pathogenicity in humans (Sandoval-Denis et al., 2016).

5. Conclusion

Our results demonstrate that BSF larvae can regularly develop on substrate supplemented with high concentrations of PVC-MPs, up to 20 % w/w, and that the size of MPs used is significantly reduced during their passage through the midgut. Only when the PVC content in the diet reached 20 %, a reduction in pupal size was observed, potentially affecting the efficiency of bioconversion, and variations in the relative abundances of some bacterial and fungal specific taxa were noted. In particular, the increase in Enterobacteriaceae and Paenibacillaceae within the midgut microbiota of treated larvae may be justified by their possible role in plastic degradation. In contrast, the reduction in the pupal size may be the combined result of multiple factors, such as the reduced amount of nutrients taken in by larvae consuming a diet containing 20 % PVC and/or simultaneous changes in the composition of the microbial community. The unknown role of the unclassified taxa, particularly high in the fungal kingdom at species level, and the function of MPs as carriers of microbiota either retained in the midgut or released in the environment, need to be further explored. Future investigations with different larval densities could also be interesting in light of comparability with real-life scenarios. This is because larval density is a key parameter in BSF rearing, significantly affecting insect behavior as well as physicochemical and microbiological dynamics. These aspects are still unclear and present promising opportunities for further investigations, especially in light of the use of BSF larvae in municipal solid waste management (organic fraction) and the use of the residual frass as an agricultural amendment.

Statements and Declarations.

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CRediT authorship contribution statement

Silvana Piersanti: Writing – original draft, Validation, Supervision, Methodology, Investigation, Conceptualization. **Manuela Rebora:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Conceptualization. **Benedetta Turchetti:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization. **Gianandrea Salerno:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Mario Ruscetta:** Investigation. **Laura Zucconi:** Writing – review & editing, Validation, Resources, Investigation. **Federica D'Alò:** Writing – review & editing, Resources, Investigation. **Pietro Buzzini:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization. **Ciro Sannino:** Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2024.06.021.

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