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Review

Flying toward a plastic-free world: Can *Drosophila* serve as a model organism to develop new strategies of plastic waste management?

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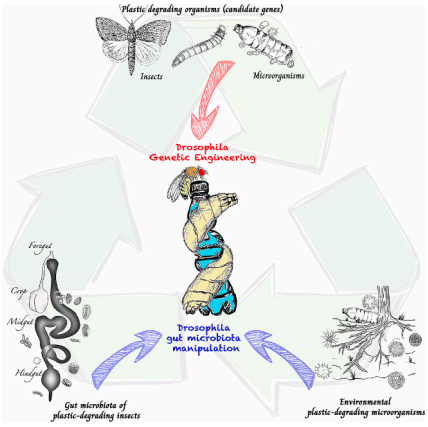
HIGHLIGHTS

- Current plastic waste management protocols can be implemented with complementary strategies.
- Insects are increasingly recognized as valuable agents in the battle against plastic pollution.
- Genome and microbiome manipulation can be applied to generate new degrader insects.
- Model organisms with no plastic-degrading capacity can be turned into degraders.
- Issues associated to the use of insects as plastic-degraders are discussed.

GRAPHICAL ABSTRACT



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ABSTRACT

The last century was dominated by the widespread use of plastics, both in terms of invention and increased usage. The environmental challenge we currently face is not just about reducing plastic usage but finding new ways to manage plastic waste. Recycling is growing but remains a small part of the solution. There is increasing focus on studying organisms and processes that can break down plastics, offering a modern approach to addressing the environmental crisis. Here, we provide an overview of the organisms associated with plastics biodegradation, and we explore the potential of harnessing and integrating their genetic and biochemical features into a single organism, such as *Drosophila melanogaster*. The remarkable genetic engineering and microbiota manipulation tools available for this organism suggest that multiple features could be amalgamated and modeled in the fruit fly. We outline feasible genetic engineering and gut microbiome engraftment strategies to develop a new class of plastic-degrading organisms and discuss of both the potential benefits and the limitations of developing such engineered *Drosophila melanogaster* strains.

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1. Introduction

Plastic polymers play a fundamental role in all productive sectors thanks to their versatility, cost, and accessibility. In developing countries, plastic polymers have rapidly and increasingly replaced paper, metals, wood, and glass, demonstrating their versatility (Andrady and Neal, 2009). Worldwide industrial processes use almost 400 Million Metric Tons of plastics every year (Zhang et al., 2022a) growing at a rate of 2.5 % per year (Plastics Europe, 2022). However, efficient disposal methods have not kept pace with the rising plastic applications. Currently, end-of-life management of plastic waste involves three scenarios: energy recovery, recycling, and landfill. However, the latter accounts for only 25 % of collected plastics (Plastics Europe, 2022). From 1950 to 2015 over 8600 MMT of plastic polymers, synthetic fiber and additives was landfilled or discarded (Qin et al., 2021) representing an unsustainable trend for the future. Environmental pollution has been documented since the early 1970s (Carpenter and Smith, 1972) leading to the destruction of marine and terrestrial habitats (Barnes et al., 2009) and the introduction of plastics into the food chain (Waring et al., 2018; Toussaint et al., 2019). While chemical and physical processes can degrade plastic waste (Yousif and Haddad, 2013; Anuar Sharuddin et al., 2016; Zhang et al., 2021) there is an increasing interest in biodegradation processes, as more organisms demonstrate the ability to degrade and metabolize plastic polymers, mainly bacteria and fungi (Lomwongsopon and Varrone, 2022), which can become instrumental in employing diverse waste management strategies.

In this review, we integrate molecular genetics, chemistry, and entomology concepts and methods to envision a perspective field of investigation that is aimed at the safe and efficient development of transgenic organisms that will hopefully aid to solve the plastic waste management and recycling problem. Here we discuss on how the current genetic engineering tools could be applied to a well-studied model organism, *Drosophila melanogaster*, to obtain a safe and efficient plastic biodegrader that conjugates the degrading ability displayed by unrelated organisms (genetic modification using genes related to plastic degradation identified in other insects and/or fungi and gut microbiota modification with plastic degrading bacteria) to the versatility of the fruit fly (i.e., easy rearing techniques, fast growth). We will discuss the benefits and the weaknesses of such an approach in the light of the current relevant literature.

2. Types of plastic

Plastic polymers exhibit a wide range of physical and chemical characteristics and are ultimately classified into thermosets and thermoplastics, based on their behavior in response to molding (Bîrcă et al., 2019). Thermosets (e.g. Bakelite, epoxy resin, silicone) irreversibly harden after polymerization, while thermoplastics (e.g. Polyethylene, PE; PolyEthylene Terephtalate, PET; PolyPropilene, PP; PolyStyrene, PS; PolyVinyl Chloride, PVC; PolyUrethane, PU) can be repeatedly molded when exposed to high temperatures. Furthermore, thermoplastics consist of monomers arranged in long chains held together by weak chemical interactions, while thermoset polymers form a strong tridimensional web of covalent bonds. Thermoplastics are produced in larger quantity compared to thermoset plastics and they are recyclable with a rate between 19 % for PET and 1 % for PP and PS (Rahimi and García, 2017).

Considering the chemical structure and the polymerization process, we can further distinguish thermoplastic polymers containing a carbon-carbon backbone, from those containing heteroatoms in their main chain (Wang et al., 2019). The first group includes polyolefins such as PE and PP as well as other addition polymers like PS and PVC. The second group includes polyesters, like PET and PU and other condensation polymers (Fukukawa and Ueda, 2012). This distinction holds significant importance regarding degradation pathways, as they are strongly influenced by the chemical structure of the polymer (Gewert et al., 2015). Condensation polymers (polyesters) hydrolyze rapidly compared to the oxidative degradation of addition polymers (polyolefins). In nature, degradation and biodegradation spontaneous processes are faster for condensation than for addition polymers (Swift et al., 2009).

3. Plastic biodegradation

Both biotic and abiotic pathways contribute to plastic degradation processes; usually, biodegradation occurs after an abiotic predegradation step (i.e., hydrolysis, UV radiation, moderate or high temperatures, or the mechanical action of complex animals) (Watling, 1993).

Industrial biotechnology mostly relies on using microorganisms (Nielsen et al., 2022). Due to its carbon-rich polymer characteristics, plastic wastes can be considered a potential source of carbon for industrial biotechnology. Although the molecular mechanisms underlying biodegradation are not yet clear for all plastic polymers, the list of organisms (bacteria, yeasts, algae, and insects) that could actively perform this process is keep growing.

The fascinating concept underlying biodegradation is the idea that utilizing carbon derived from plastic could close the waste cycle, leading to the production of biomass applicable in various economically relevant sectors such as agriculture (fertilizer production) and the energy field (waste-to-energy conversion and biomass production) (Beale et al., 2022). Given their potential utilization in many economic fields, including agriculture (Poveda, 2021), fisheries (Alfiko et al., 2022), and human nutrition (Nowakowski et al., 2022), insects are excellent candidates to develop new recycling strategies and models of circular economy.

4. Plastic-degrading organisms: where do we start from?

The current scientific literature provides substantial evidence that biodegradation has at least two non-negligible limitations that dampen enthusiasm, despite its low-cost and eco-sustainability: the time required for degradation and the limited knowledge we have acquired so far in the characterization of the different pathways involved in plastic degradation.

Despite the knowledge of eighty-nine organisms (Supplemental Table 1, Fig. 1) capable of degrading PE, the polymer biodegradation time rate remains significantly longer than that of established methods (Zhao et al., 2020a). Bio-cultures of single unicellular plastic-degrading microorganisms take too long to effectively perform their bioremediation function, i.e. *Stenotrophomonas* sp. *P2* and *Achromobacter* sp. *DF22* isolated from waste dumpsite and drilling fluid form biofilm after 30 days when cultured in basal medium containing LDPE film as the sole carbon source. Microscopy and spectroscopy analyses revealed both the erosion and the presence of new functional groups on the treated plastic surface (Dey et al., 2020).

Also, the combination of different microorganisms, i.e. a consortium of fungi consisting of *Aspergillus niger*, *Aspergillus flavus,* and *Aspergillus oryzae*, cooperatively leads to LDPE surface erosion suggesting that communities may perform better than single organisms in biodegradative processes, although it still requires more than 50 days (GC et al., 2021) for degradation.

Thirty different prokaryotic species and six insects have been shown to degrade PS (Supplemental Table 1, Fig. 1), most of which suffer from the same time limitation described above. Species of the genera *Bacillus* and *Exiguobacterium* are able to degrade up to 34 % of PS within 60 days of incubation (Auta et al., 2017a; Chauhan et al., 2018; Ganesh Kumar et al., 2021).

The chlorinated nature of PVC makes it difficult to degrade via microbial activity. However, a small number of microorganisms (Supplemental Table 1, Fig. 1) can modify PVC substrates over a three months incubation periods (Kumari et al., 2019). Biodegradative processes targeting other plastics suffers from the same limitation, as demonstrated

by studies on PP degradation using *Bacillus flexus* and *Pseudomonas azotoformans* (22.7 % weight loss after one year incubation on PP (Aravinthan et al., 2016)) or *Bacillus cereus* and *Sporosarcina globispora* (11 % weight loss after 40 days of incubation on PP (Auta et al., 2017b)). Insects also take a long time to degrade plastics, since 300 *T. molitor* and 200 *Z. atratus* larvae fed with PP foam as sole diet need up to 35 days to consume 1–3 mg of PP (Yang et al., 2021a).

The second main limitation consists in the poor characterization of the enzymatic pathways, and their associated genes, from plastic-degrading organisms. As can be observed (Fig. 1), a long list of plastic-degrading organisms (Supplemental Table 1) does not match an equally long list of characterized enzymes and genes (Supplemental Table 2). To date, we have an advanced knowledge of the key enzymes involved in PET biodegradation. The enzymes responsible of PET degradation have been isolated or characterized in 43 PET degrading species (Supplemental Table 2, Fig. 1). The best characterized enzyme is PETase, an esterase first identified in *Ideonella sakaiensis* (Seo et al., 2019). Other hydrolases, including lipases, cutinases, and esterases have

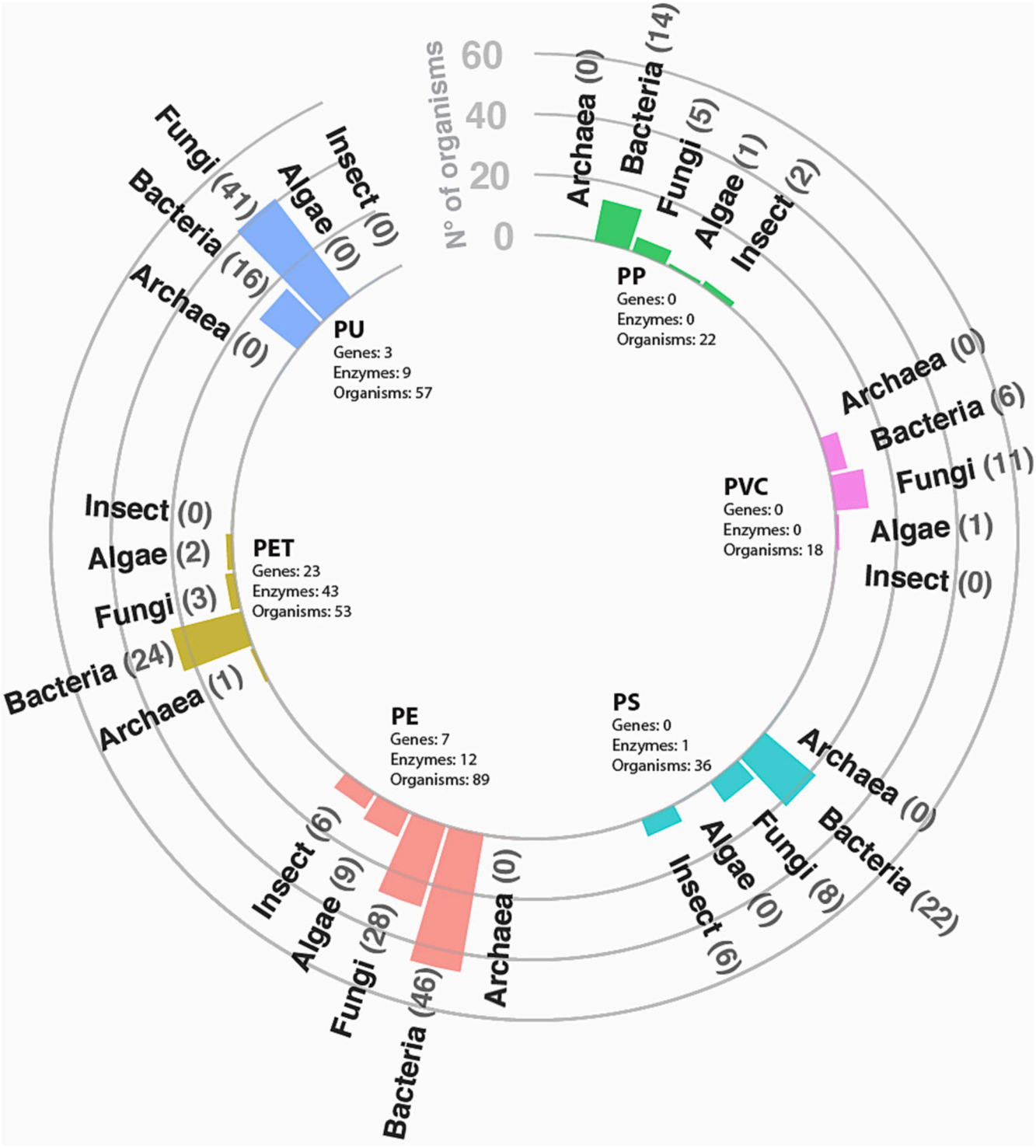

Fig. 1. Summary of the relative abundance of plastic-degrading organisms (see Supplemental Table 1 for further details). The number of plastic-degrading organism is shown in parentheses. The number of known genes, enzymes characterized, and total number of plastic-degrading organisms is specified in the legend.

been characterized from the most different PET degrading organisms. Almost the same enzymatic activities isolated from other microorganisms (7 bacteria, 2 fungi) are responsible for the degradation of PU (Supplemental Table 2, Fig. 1).

Our understanding of the biochemical basis of PE biodegradation has also experienced a marked advancement in the recent years. The alkane monooxygenase system of *Pseudomonas aeruginosa* has been widely characterized (Wilkes and Aristilde, 2017), along with the identification of other enzymes expressed by PE degrading bacteria and fungi. Transcriptomic studies in *Rhodococcus ruber* cultivated on PE powder have revealed the up-regulation of alkane and fatty acid degradation pathways, supporting the hypothesis that the Alkane Hydroxylase system could be involved in PE degradation, and that the products can enter the fatty acid metabolic pathways (Gravouil et al., 2017). Notably, insect enzymes (further described in the following paragraphs) have recently been identified as the main degrading enzymes isolated in the saliva of *Galleria mellonella* (Sanluis-Verdes et al., 2022). It is worth noting that the genetic basis of plastic degradation is very poorly understood, since few genes have been identified compared to the number of known enzymes (Supplemental Table 2, Fig. 1).

5. Where we are going: insects as an unexpected source of plastic-degraders

As mentioned above, research on plastic degradation is moving on the use of microorganisms' consortia, rather than single species (Skariyachan et al., 2019; Cao et al., 2022). Complex eukaryotes, such as animals, are natural bioreactors where microbial communities constitute part of a super-organism, the holobiont (Margulis and Margulis, 1993), composed by the host plus microbial partners-in-health. Several anatomical districts host complex ecosystems that together with the host tissues provide the conditions for fulfilling the physiological functions. The gut is the best-studied animal organ that provide an example of the intimate contact between the gut flora and the host tissues. In this view, Arthropods belonging to the Insect Class can represent a unique opportunity to identify excellent plastic degraders. Indeed, given the similarity between the chemical structure of PE and waxes (LeMoine et al., 2020), it is conceivable that parasites of honeybees could also be used for plastic degradation. Many organisms produce alkane-derived compounds as waste products, a structural element, a defense mechanism, or as chemoattractants (Rojo, 2005). As an example, beeswax is a complex mixture of more than 300 organic compounds (Tulloch, 1980). Several studies have reported that larvae feeding on honeycombs' wax also alter PE. It has been recently observed that PE shopping bags left in contact with living wax worms (*G. mellonella*) showed visible holes (Bombelli et al., 2017; Billen et al., 2020). Similarly, the lesser wax worms (*Achroia grisella*) make holes when left in contact with a HDPE (Kundungal et al., 2019). Moreover, *T. molitor* larvae, can chew and eat LDPE foam (Yang et al., 2021b). SEM analysis of the treated PE surface showed physical changes in the polymer after incubation with *T. molitor* larvae (Bulak et al., 2021). In addition to natural wax-feeding insects, it has been noted that other invertebrates belonging to different Orders of Insects may degrade plastics, including Lepidoptera (*Plodia interpunctella*) and Coleoptera (*Zophobas atratus*, *Tenebrio obscurus*, *Tribolium castaneum*, *Tribolium confusum*, *Plesiophthalmus davidis*, and *Uloma* sp.) (Yang et al., 2022). To a lesser extent, other insects could thrive on plastics such as the midge *Chironomus sancticaroli,* which is able to accumulate plastic in its gut (Palacio-Cortés et al., 2022), and *Spodoptera frugiperda*, which can survive feeding on PVC films (Zhang et al., 2022b).

6. Role of the insect gut microbiota in plastic degradation

Insects, like other organisms, harbor a complex microbiota that lives in and on their bodies. A classic example of the importance of microbes for the degradation of complex polymers is their role within the gut of higher termites, where the lignocellulose part of the plant material they ingest is mostly degraded by the cellulolytic activity of microbial symbionts (Zhang et al., 2023). Similar lignocellulose degradation has been also associated to the gut microbiota hosted by phytophagous and xylophagous insects belonging to the Orders Coleoptera, Blattodea, and Orthoptera (Gales et al., 2018). The gut microbiota could therefore reasonably play a similar role in plastic-degrading insects. Most of the studies on plastic-degrading insects and their microbiota have been carried out in three organisms: *T. molitor*, *G. mellonella* and *Z. atratus*. However, it remains unclear whether the ability of these insects to degrade plastic polymers is due to their gut microbes, the host's enzymatic repertoire, or both, leading to ongoing debates.

Usually, the composition of the gut microbiota in insects can change over time due to environmental conditions, nutrient availability, growth stage, and health status (Berg et al., 2020). Studies analyzing microbial communities in the insect gut have yielded contrasting results (Supplemental Table 3). For instance, supplementing *G. mellonella*'s diet with LDPE (1:28 ratio) for 5–7 days did not significantly alter of its bacterial community composition (Noel et al., 2022). However, different experimental setups, revealed a wide spectrum of bacterial genera positively correlated with PE consumption (Supplemental Table 3). Using a higher amount of plastic supplementation to *G. mellonella* diet [1:1 plastic (PE/PP/EP):honeybee wax] for 8 days elicited an increase of *Desulfovibrio vulgaris* and *Enterobacter* relative abundance with the PE diet while PS and PP lead to an increase of *Enterococcus* relative abundance (Peydaei et al., 2021). In a longer trial (21 days), feeding *G. mellonella* larvae with PE or PS resulted in an increased abundance of *B. cereus* and *Serratia marcescens* compared to a bran diet. Although feeding caterpillars with plastic only gave the best plastic degranulation results, a plastic:honeybee wax co-diet reduced plastic consumption while improving gut microbial diversity and larvae survivability. These factors should be also considered for the development of plastic degradation/rearing bioreactor (Lou et al., 2020).

In *Z. atratus* larvae, PE feeding alters the gut microbiota. A strong association between PE diet (35 days) and *Citrobacter* was demonstrated together with a higher relative abundance of *Enterococcus* sp. in PE fed larvae gut against bran-fed larvae gut (Luo et al., 2021). *Citrobacter* sp. and *Kosakonia* sp. are found at significantly higher relative abundance in the gut of *T. molitor* larvae fed on PE or PS compared to the bran-fed control (32 days experiment). Both belong to the Enterobacteriaceae family and are aerobic or facultatively anaerobic bacteria, suggesting that a metabolic pathway which requires oxygen incorporation may facilitate plastic degradation (Brandon et al., 2018). *T. molitor* also showed the highest growth capability on different kind of plastic, including PS foam. Co-diets approaches (PS:bran at different ratios) highlighted the importance of water for the biodegradation process and its impact on the gut microbiome (Tsochatzis et al., 2021a).

Multiple studies confirmed the essential role of insect gut microbiota by isolating commensal strains from larvae fed exclusively on PE or on a PE-enriched diet (Yang et al., 2014; Ren et al., 2019; Cassone et al., 1922; Montazer et al., 2021) and testing them to grow on PE. PS degrading bacterial strains have been mostly isolated form *T. molitor*: i) *Exiguobacterium* sp. strain YT2 (Yang et al., 2015). ii) Three different Gammaproteobacteria *Klebsiella oxytoca* strain 9A, *P. aeruginosa* strain Ki2y and *S. marcescens* strain 6 A (Urbanek et al., 2020).

P. aeruginosa strain DSM 50071 isolated from *Z. atratus* degrades PS, probably thanks to a serine hydrolase pathway, and it is also able to degrade PE, PP and polyphenylene sulfide (Lee et al., 2020).

7. Manipulation of the insect gut microbiota

The generation of axenic individuals is an important experimental tool to investigate the role of the microbial fraction of an organism. Axenic organisms are biological entities composed of a single species and completely free of other associated (micro)organisms. Living organisms, including multicellular animals and plants, are currently viewed as complex association of multiple communities, mainly

composed of microorganisms. It follows that, axenic organisms do not exist in nature, although they can be created under controlled laboratory conditions. Axenic insects are particularly useful to study how the microbiota impacts on the immune system, the organism lifespan, and behavior (Smith et al., 2007; Brummel et al., 2004; Wong et al., 2015). Another application of axenic organisms is the generation of gnotobionts, organisms where the microbiota has been replaced or manipulated. These manipulation methods make possible to assess to what extent a different gut microbial composition reflects the plastic degradation efficiency, by directly assaying the plastic degradation products in larvae frass. Methods for generating gnotobionts are available for model (Koyle et al., 2016) and non-model insects (Houser and Burns, 1968; Jarosz, 1981).

Axenic larvae of *T. molitor* and *Z. atratus* fed on PS (Yang et al., 2015) or PE (Peng et al., 2020) as the sole food source, did not show any ability to depolymerize plastics while PE degradation products were extracted from non-axenic larvae frass. This allowed drawing conclusions on a prominent role of the intestinal microbiota in the plastic degradation process. However, recent studies on *T. molitor* and *T. obscuris* axenic larvae showed that they are capable of degrading LDPE in the absence of the gut microbiota, although the rate of degradation was lower in comparison to the untreated larvae (Yang et al., 2021b). Similar results were obtained in a 60-day experiments showing that the reducing gut microbial abundance led to a decrease, but not to the abolition, of LDPE depolymerization. In contrast, expanded biodegradation of PS and PP showed microbial dependency being completely inhibited after the antibiotic treatment, with *Kluyvera* sp. associated to PP degradation and 4–5 different OTUs associated to expanded PS degradation. *G. mellonella* germ-free larvae still successfully decompose long-chain fatty acids following wax metabolism (Kong et al., 2019). Similar results were obtained in PS-related studies, where *G. mellonella* germ-free larvae successfully depolymerized PS polymers, suggesting that it may be microbe-independent. However, metabolomic analysis showed the loss of potential styrene catabolic pathways in axenic larvae indicating that microbes may still partially contribute to PS degradation (Wang et al., 2022).

8. Why Drosophila could be used as a biotechnological tool for plastic degradation

Drosophila melanogaster is one of the most important model organisms in scientific research. Several research fields have experienced significant advancement thanks to the contribution of the fruit fly, ranging from genetics to development, from neuroscience to drug discoveries (Stephenson and Metcalfe, 2013). The use of *D. melanogaster* has many technical advantages, being easy and quite inexpensive to bred, having a shorter life cycle compared to vertebrate model organisms, and producing a large offspring allowing both classic genetics and high throughput approaches (Millet-Boureima et al., 2021). Moreover, in the last century many genetics tools to study *D. melanogaster* have been developed (Hales et al., 2015). In addition, *Drosophila* is a terrific model organism to study microbiome-host interactions (Han et al., 2017), as axenic individuals can be easily obtained (Kietz et al., 2018).

To combine the plastic degradation potential of genetically encoded functions and microbiome action, a model organism that can be fully manipulated is highly desirable. In the following paragraphs we discuss of the most relevant aspects of the biology of *D. melanogaster* together with the available molecular technologies that can lead to the hypothesis that the fruit fly can be considered as a good candidate for the development of new strategies, based on transgenic technologies, for plastic waste management.

9. Life cycle comparison of plastic-degrading insects vs *Drosophila melanogaster*

Life cycle duration is crucial for insect strain management and the application of a given organism in a process. Firstly, it directly impacts the production of the initial biomass required for the process. Additionally, the duration of individual developmental stages becomes critical since, in most cases, only a particular stage is useful for conducting the desired process. Fig. 2A and Supplemental Table 4 summarize the developmental stage duration in relevant insect species capable of degrading plastic polymers, compared to non-degrader *D. melanogaster*. However, a rapid life cycle provides a great opportunity to deploy a large number of individuals, considering that the fruit fly's egg-laying capacity is comparable to that of other insects. This is particularly advantageous when designing insect-based industrial processes.

Insects have four distinct developmental stages, embryo, larvae, pupal, and adult. Embryos and pupae are less attractive when developing insects-based strategies for plastic degradation due to their sessile nature. In turn, adults lack a robust masticatory apparatus, and most of them are aphagous during the adult stage (Gangwere, 2005). Larval stage is therefore worth of consideration also considering that larvae cannot easily escape the site of application when used as plastic degraders, an important feature that allows the biocontainment when transgenic organisms are used (see next paragraphs).

Larvae are therefore promising stage for transgenes expression thanks to their mobility and the presence of mouth hooks that facilitates solid substrate mincing, ingestion, and digestion.

Although *D. melanogaster* larvae do not have a potent masticatory apparatus if compared to lepidopteran larvae, they can be still useful to meet the requirements for plastic degradation thanks to the available genetic engineering technologies that can bring new genes into the fly genome and allow the expression of desired phenotypes.

10. Transgenic technologies in *Drosophila melanogaster*

Drosophila melanogaster (Rubin and Spradling, 1982) and the mouse (Gordon and Ruddle, 1981) emerged as pioneering eukaryotes organisms in which foreign genes have been successfully introduced Transgenic technologies are powerful tools that enable the introduction and the expression of foreign DNA sequences into the preferred organisms, thus creating exceptional experimental models for gene-associated studies. The characterization of *P-element* transposon (Bingham et al., 1982), drove the development of *Drosophila* transgenic technologies in the 1980s. In 1990s, discovery like the GAL4/UAS system in yeast (Guarente et al., 1982), enabled the conditional control of transgene transcription (Kakidani and Ptashne, 1988; Fischer et al., 1988). Transgene expression can be controlled over spatial (tissues) and temporal (development) dimensions, through enhancer-driven expression of the GAL4 transcription factor (enhancer trap line), which activates the transgene under the UAS promoter. *D. melanogaster*, boasts hundreds of enhancer trap lines, allowing expression in virtually all organs and tissues of the fly. Furthermore, *Drosophila* transgenic lines expressing the GAL80 protein, a transcriptional repressor that antagonizes GAL4, allows transgene expression in a subset of cells (expressing cell-specific genes) within a tissue or organ (Suster et al., 2004), boosting the power of transgenesis in *Drosophila*. Additionally, the implementation of sequence specific recombination systems, such as the FLP/FRT, the CRE/Lox (Frickenhaus et al., 2015), and the PhiC31 (Bateman et al., 2006) systems further improved the transgenic studies in *Drosophila*. These systems allow transgene integration in genomic loci far from interfering loci, minimizing or eliminating position effects on the phenotype resulting from random transgene integration. This feature is

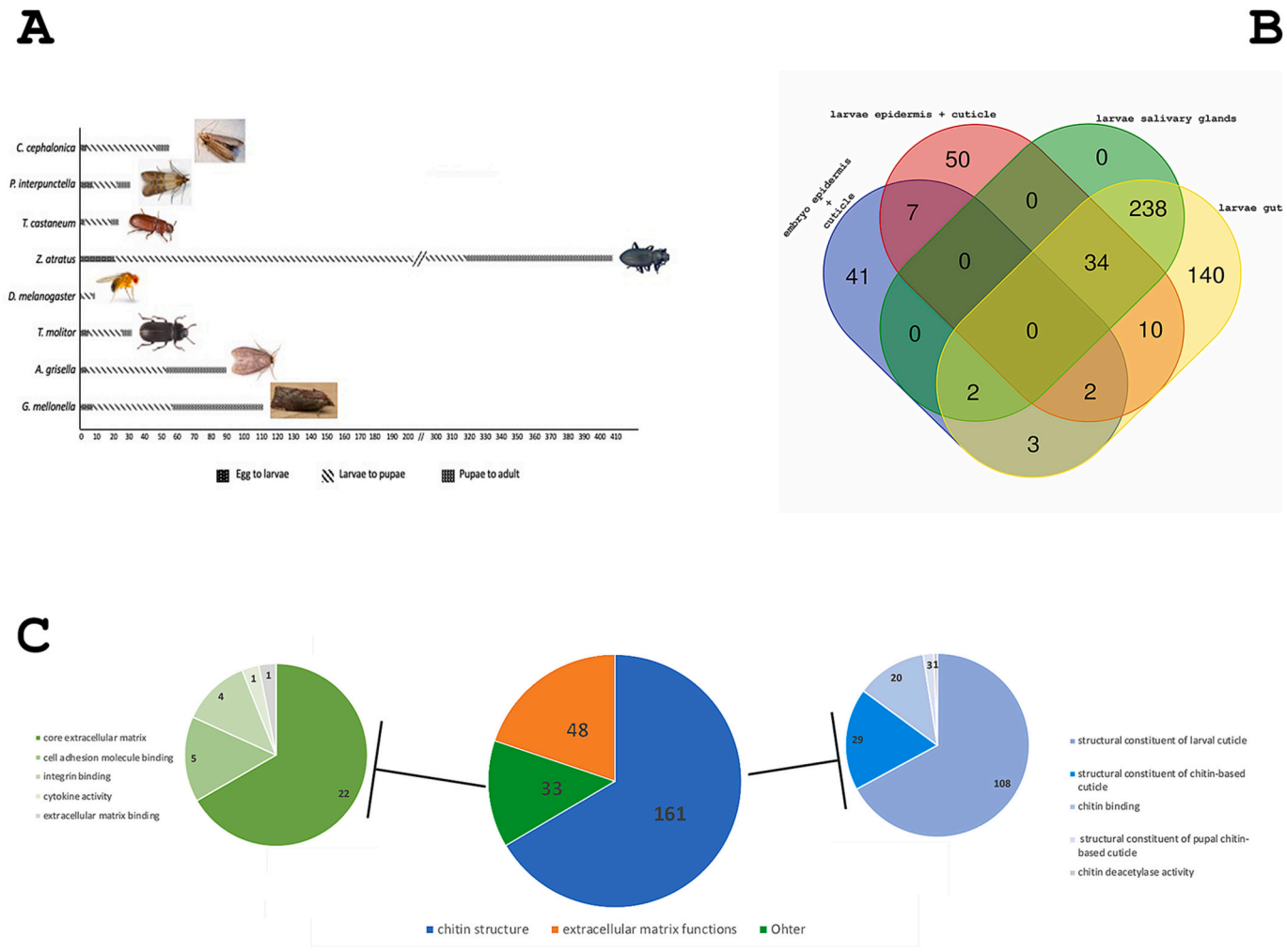

Fig. 2. A) The developmental stage duration of plastic-degrading insects compared with the non-degrader *D. melanogaster*. B) Venn diagram summarizing the tissue specificity of the GAL4 expression drivers available from the Bloomington Stock center (Supplemental Table 4, last database accession March 2023). C) Overview of the annotated secreted proteins of *D. melanogaster* categorized according to the associated GO terms.

particularly desirable when the effect of specific transgene subsequences is studied since an easy removal of specifically designed sequences from the transgene allows downstream studies without changing the transgene map position (Conte et al., 2002). Finally, genome editing technologies in the *Drosophila* molecular genetics' toolkit enable precise genome modifications including generating new alleles (Port et al., 2014), inserting foreign genes and their associated regulatory regions, and defining precise expression patterns (Lin et al., 2015). These advancements owe much to over a century of genetic research, resulting in a variety of mutants still employed in laboratory practice. Balancer chromosomes (Miller et al., 2019) are one of the most powerful tools in fly genetics. They facilitate progeny selection when crossing individuals carrying balancers, thanks to the presence of both dominant and homozygous lethal genetic markers. Beyond maintaining lethal mutations or transgenes as heterozygous populations, balancer chromosomes are fundamental tools in the construction of complex genotypes (Roote and Prokop, 2013). The versatility offered by *D. melanogaster* in terms of knowledge, standardized protocols, and time and cost effectiveness, suggest that investing in *D. melanogaster* as a model organism can lead to the development of new and efficient bioremediation strategies.

11. Can we turn *D. melanogaster* into a transgenic plastic degrader?

As mentioned earlier, characterizing plastic-degrading microorganisms involves defining the genes responsible for driving the degradation process. However, this is a challenging task that often requires complex mutational screening or advanced technologies. The number of known plastic-degrading organisms exceeds the number of characterized enzymes, highlighting the difficulty in identifying these enzymes and their associated genes (Supplemental Tables 1 and 2). Gene identification requires the complete assembled genome sequence of the organism, a particularly complex task in eukaryotes due to their large genome size, which can subsequently enable gene characterization and its expression pattern.

Recently, plastic-degrading enzymes have been identified and described in insects. The saliva of *G. mellonella* is capable of PE oxidation and depolymerization (Sanluis-Verdes et al., 2022) thanks to enzymes belonging to the hexamerin superfamily and Arylphorin family (Hughes, 1999), whose structure and molecular organization has been recently resolved (Spínola-Amilibia et al., n.d.). These proteins are phylogenetically related to phenol-oxidases (enzymes targeting aromatic rings) and hemocyanins, oxygen transport proteins, which exhibit phenol oxidase activity (Glazer et al., 2013). To our knowledge, this represents the first

report of a gene product in a higher eukaryote capable of plastic degradation, holding potential for novel strategies in plastic waste management using transgenic organisms. Although *D. melanogaster* possesses homologs of the arylphorin encoding genes (Burmester, 1999) it cannot oxidize PE, highlighting the functional diversification of arylphorin-like proteins. The availability of the genome assembly of *G. mellonella* (Lange et al., 2018) allows an easy identification of the gene that encode these proteins, thus enabling both gene expression studies and the opportunity to transfer these genes into new organisms through the available transgenic technologies. Moreover, the identification of insect-degrading proteins in *G. mellonella* suggests the possibility of finding functional homologs in other polyethylene-degrading insects.

12. Transgene expression strategies in *Drosophila melanogaster*

All the genes listed in Supplemental Table 2 can be ectopically expressed in *D. melanogaster*, but issues arise when expressing a transgene. One concern is the potential lethality or semi-lethality associated with high transgene product dosage, even if the expressed protein has no homologous functions in the host. For instance, overexpressing the tetracycline trans-activator gene in mosquitoes led to a late larval/pupal stage lethality (Knudsen et al., 2020) due to "transcriptional squelching" (Gong et al., 2005). Therefore, a strong constitutive transgene expression it is not always recommended (Lee et al., 1998).

Weak constitutive promoters such as those derived from transposable elements (Palazzo and Marsano, 2021) offer a possible alternative overexpression strategy, enabling low and constitutive levels of transcription in a tissue-independent manner, thereby improving the tolerance of ectopically expressed proteins. Rather, expression patterns dictated by tissue specific promoters can overcome this issue. Another concern is related to transgenic product bioavailability in anatomical districts that can become in contact with the substrate.

As justified above, insect larval stage is prime choice for designing insect-mediated plastic degradation strategies. In this context, larvae of *D. melanogaster* can be considered as an added value since they can express transgenes virtually in all anatomical districts. Large collections of transgenic enhancer trap lines expressing the GAL4 transcription factor can be used to drive the expression of transgenes under the control of a UAS promoter. This approach can be employed in enterocytes, salivary glands or in the cuticle, in order to maximize the transgenic protein bioavailability in plastic-degrading strategies based on transgenic insects. Overlapping GAL4 lines expression can also increase the transgene expression spectrum in *D. melanogaster* (Fig. 2B and Supplemental Table 5). In addition, incorporating secretion peptides to enable extracellular protein release, further enhances the expression strategy impact. Over 250 known *D. melanogaster* genes encode secreted proteins (Supplemental Table 6 and Fig. 2C) offering a repertoire of secretory signal peptides for fusion with the protein of interest. In a recent paper by Clark et al. (Clark et al., 2022) demonstrated protein expression in secreted form in *D. melanogaster* using laccase genes from fungal species *Trametes trogii* and *Polyporus brumalis*. They replaced the fungal laccases' leader peptides with the signal peptide of the *D. melanogaster clp9* gene. Both transgenes were expressed under the short tubulin promoter to mitigate potential toxic effects of transgene overexpression (Waters et al., 2018). One of the transgenic strains was able to degrade the laccase substrates ABTS both at the larval and the adult stages in vivo. Also, lyophilized powder obtained from adults can be used to degrade BPA and indigo carmine, two aquatic pollutants (Clark et al., 2022).

The larval salivary glands are relevant organs for achieving transgene expression to develop plastic-degrading transgenic flies. They are anatomically connected to the external environment through the larvae mouth and play a vital role in of *D. melanogaster* larvae. Salivary glands secrete a complex mixture of glycosylated proteins called glue, which is expectorate and spread over the whole larvae body, attaching pupae to the substrate during the late-larval stage (Fraenkel and Brookes, 1953). Additionally, they secrete glycosylated mucin or nondigestive enzymes that aid in food lubrication throughout the larvae life (Costantino et al., 2008). Therefore, larval saliva becomes as an excellent fluid to spread the recombinant proteins expressed by transgenes onto the plastic substrate, also taking advantage of the larvae movement.

The larval gut stands out as the most promising organ for achieving transgene expression for plastic degradation. Advantageously, this organ enables a double expression strategy of transgenes expressing plastic-degrading enzymes Specific GAL4 enhancer traps allow the targeted expression in this organ. Furthermore, since the gut hosts a diverse and dynamic community of microorganisms, it could be exploited as a bioreactor into which plastic-degrading microorganisms can be grown or engrafted. Numerous studies have elucidated that the composition, of the *Drosophila* gut microbiota is extremely variable. 90 % of the observed species belong to three orders (Enterobacteriales, Rhodospirillales and Lactobacillales) (Chandler et al., 2011) and five families (Acetobacteraceae, Lactobacillaceae, Leuconostocaceae, Enterobacteriaceae, Enterococcaceae) (Staubach et al., 2013). Gut microbiota varies both in composition and relative abundance of taxa within and among *Drosophila* populations in relation to diet and the surrounding environment (Broderick and Lemaitre, 2012; Wong et al., 2013). *Drosophila* embryos are sterile, and bacteria that colonize larval gut are acquired from adult feces (Broderick and Lemaitre, 2012). These features represent an opportunity to generate gnotobiotic strains, in which the intestinal microbiota has been engrafted with plastic-degrading bacteria or engineered bacterial strains ectopically expressing key degrading enzymes.

13. Relevant issues in insect-mediated plastic biodegradation

13.1. Standardization of the methods for measuring biodegradation ability

Studies focused on organisms capable of using plastic polymers as a sole carbon source have been crucial to understand the biological basis of plastic biodegradation and are mandatory to identify novel strategies of bioremediation. However, how this capacity should be measured seems to be a point of confusion, standing to the many different methods used so far. Many works make use of gravimetric methods to prove plastic consumption and animal weight preservation, but this is far from proving plastic metabolization. Since this is at the basis of recycling and circularity approaches, it is important to follow standardized procedures when plastic degradation is evaluated.

We propose that a consistent and standardized methodology should be applied to establish if the carbon structure of the plastic polymer is modified by the action of the insect and to assess its entrance into the animal's metabolism. To this purpose, Nuclear Magnetic Resonance (NMR) and Fourier Transform-Infrared spectroscopy (FTIR) can be considered as the gold standards to determine quantitatively and qualitatively the plastic substrate modifications that occurred after the treatment with the degrading organism. Measurements can be performed on different types of specimens obtained from the digestive and excretory tracts and accessory organs, such as homogenates of salivary glands and gut tissues or filtered stool extracts (Fig. 3).

FTIR spectroscopy is one of the best methods to investigate molecular structure and bonds based on their characteristic vibrational frequencies. For instance, the FTIR spectrum of HDPE incubated with the fungus *A. flavus*, isolated from the gut of *G. mellonella*, shows the appearance of new adsorption peaks compared to the control (without exposure to *Aspergillus*). The broad peaks in the range of 3500 to 3100 cm^{-1} correspond to hydroxyl groups (OH) and those at 1113 and 1647–1716 cm^{-1} are attributed to ether groups (C-O-C) and carbonyl groups (-C=O), respectively, thus indicating the bio-oxidation of the plastic substrate (Zhang et al., 2020). Moreover, FTIR spectra of PE coupons treated with homogenized salivary glands showed the formation of degradation intermediates including carbonyl groups, suggesting

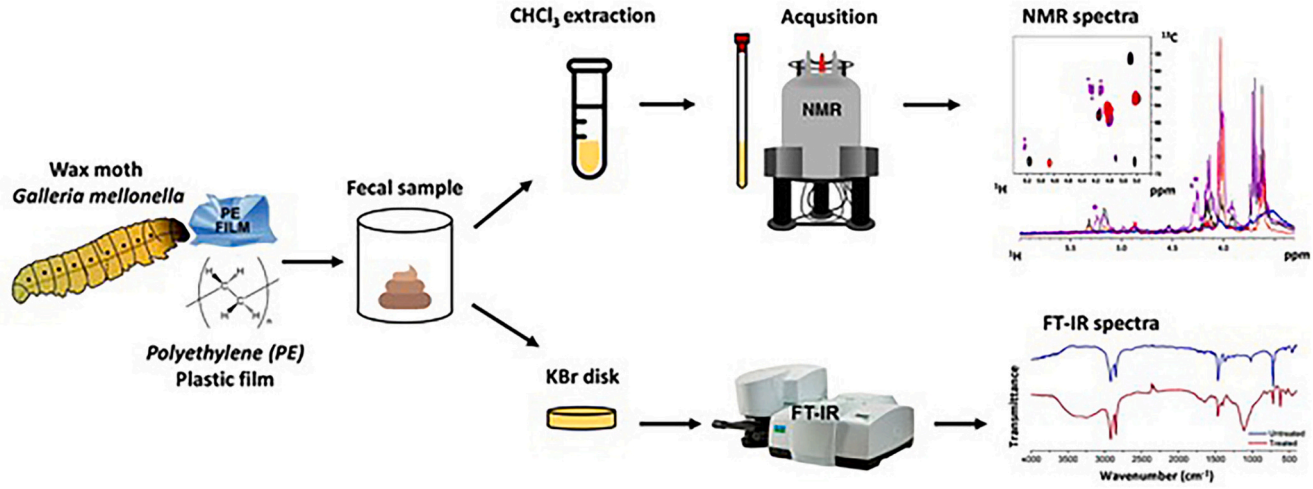

Fig. 3. Schematic workflow of sample preparation and spectroscopic analysis of feces from PE-degrading larvae of *G. mellonella*.

that wax moth salivary glands might aid in PE degradation (Peydaei et al., 2020). The formation of new FTIR peaks in *Galleria*-treated PE was primarily related to changes in fatty acid composition, not unlike those observed in beeswax degradation, due to the structural similarity between wax and PE (Kong et al., 2019). FTIR samples can be analyzed either by preparing solid pellets with a purified salt (usually potassium bromide) or directly in the liquid or homogenized state, without further treatment, when the FTIR spectrometer is equipped with attenuated total reflectance (ATR) sampling accessory. Although FTIR spectroscopy helps identify the type and content of functional groups and is a quick and easy experiment, the accuracy and resolution are still not high.

NMR spectroscopy is another suitable technique for monitoring the biodegradation of plastics and, in general, for metabolomics studies. A distinctive feature of NMR is the sensitivity of the chemical shift of an NMR active nucleus to changes in its chemical environment. NMR allows for the simultaneous detection of various metabolites from a broad range of chemical classes, including aminoacids, short-chain fatty acids, amines, and alcohols. With the use of NMR, compounds present at concentrations as low as μM in excreta residue (egested frass) can be detected and quantified with a single measurement and relatively simple sample preparation. 1D ^{1}H NMR spectroscopy provided evidence of plastic mineralization by waxworm and mealworm, supported by the reduction/disappearance of the peak at 1.3 ppm assigned to PE alkyl protons (Kundungal et al., 2021) and by the appearance of a new peak at 5.3 ppm in a region associated with alkene bonds (C=C-H), indicating oxidation and cleavage of PE chains (Brandon et al., 2018). Two-dimensional (2D) NMR spectroscopy can be applied to reduce signal overlap and better identify plastic degradation metabolites. A particularly useful 2D NMR method is ^{1}H—^{13}C HSQC (heteronuclear single quantum coherence spectroscopy), which allows the detection of any ^{13}C atom directly bound to a proton, thus taking advantage of the much wider chemical shift range of ^{13}C (Dona et al., 2016).

Moreover, the use of ^{13}C-labeled substrates can reveal whether a polymer structure is assimilated and incorporated into biological molecules (Chokkathukalam et al., 2014). Using solid-state ^{13}C NMR spectroscopy and gas chromatography coupled to mass spectrometry (GC–MS), the cleavage of ^{13}C-labeled PS molecules and the formation of depolymerized metabolites in the larval gut of *T. molitor* was demonstrated (Yang et al., 2015). Furthermore, it was shown that ^{13}C-labeled PS was partially biomineralized to $^{13}CO_2$ and the remaining ^{13}C was incorporated into lipids. To this end, the extracted derivatized fatty acids were separated by GC and analyzed by combustion−isotope ratio MS, which gave the $^{13}C/^{12}C$ ratio (expressed as $\delta^{13}C$) for each individual component (Hoffman and Rasmussen, 2022). While GC–MS helped detect volatile, non-polar compounds based on their retention times, solid-state ^{13}C NMR spectroscopy was applied to identify the native composition of the solid substrate (excreted in the larval feces) without separation of the components (Yang et al., 2015).

Although MS is highly sensitive and one of the most common analytical techniques for metabolomics studies, solution and solid-state NMR spectroscopy can exhibit greater reproducibility and easier sample preparation. In addition to increasingly higher magnetic fields, the availability of cryogenically cooled probes improves NMR sensitivity, thus enabling the detection of naturally abundant ^{13}C without isotope enrichment. Simple NMR-based metabolomics workflows have been reported in the literature for studying gastrointestinal diseases such as ulcerative colitis (UC), irritable bowel syndrome (IBS), Crohn's disease, and helminth infections (Kostidis et al., 2017; Bjerrum et al., 2015). Similar approaches have been applied to fecal samples of plastic-degrading insects to understand polymer degradation and metabolism (Kim et al., 1730; Pivato et al., 2022).

13.2. Generation of byproducts and microplastic

To maximize the overall process efficiency, two critical aspects must be taken into consideration when utilizing insects to address the plastic waste problem: the plastics amount each individual insect can degrade and the generation of by-products. While it is possible to achieve the overexpression of plastics-degrading enzymes in transgenic insects, this approach may also lead to developmental issues and lifespan impairment (Knudsen et al., 2020; Gong et al., 2005; Lee et al., 1998). The latter issue could be compensated by generating more insect biomass.

A more pressing concern arises from byproducts management. Complete insect-mediated biodegradation of plastics ideally involves enzymatic transformation that can also include bio-assimilation, meaning that carbon atoms from plastic polymers enter the insect metabolism. Bio-assimilation has been rarely described (Yang et al., 2015), whereas plastics oligomers have been frequently detected in the intestines and feces of larvae fed with plastics, along with the presence of micrometric plastic debris (Rejasse et al., 2022; Tsochatzis et al., 2021b). These findings suggest that byproducts may arise through biochemical modifications or mechanical actions by insects. Addressing the first issue, further research should focus on understanding the metabolic pathways involved in the degradation process. The second issue pertains to insects with strong mandibular apparatus capable of grinding plastic substrates. While this grinding ability can enhance insect-mediated biodegradation by increasing the enzyme/substrate contact surface, it also raises concerns about the generation of microplastics. According to the National Oceanic and the European Chemicals Agency, microplastic is defined as plastic particles in the range of 0.5 to

5 mm in length (Lebreton et al., 2018). Typically, microplastics result from the fragmentation of larger plastic materials by weathering agents. Once released into the environment, they can be carried far from their source by winds and ocean currents, ultimately reaching remote regions like the deep sea and polar caps. Microplastics are inadvertently consumed by zooplankton, fish, birds, and marine mammals, and they can also enter the human body through respiration or contaminated food. Although most ingested plastics are excreted from the body, a small fraction is absorbed. Microplastics may release toxic compounds, including plasticizers used to enhance physical resistance in plastic polymers, which have been shown to act as endocrine disruptors in animals (Khan et al., 2022). Experiments on mice have demonstrated that ingestion of microplastics can lead to oxidative stress, impairment of the gut's epithelium permeability, and inflammatory intestinal imbalance (Hirt and Body-Malapel, 2020). In *D. melanogaster*, microplastics have strong impact both on physiology (Shen et al., 2021) and gene expression (Alaraby et al., 2023). Data on humans are limited but, depending on the routes of exposure and types, microplastics might cause adverse effects on gastrointestinal cells, airway cells, and immune cells (Banerjee and Shelver, 2021). Experiments conducted using organoids as experimental systems confirm the toxic effect of microplastics on human health (Cheng et al., 2023). Microplastic-mediated tissue damage should be therefore considered when designing plastic-degrading transgenic insects. Also, to mitigate the environmental impact, future industrial plants for insect-based plastics biodegradation should be equipped with air filters and wastewater treatment technologies, such as membranes that can retain plastic microparticles (Khan et al., 2022).

13.3. Reproducibility issues in insect-mediated plastic degradation experiments

Independent studies on the role of the gut microbiome of plastic-degrading insects, often yielded inconsistent results due to the lack of standardized methods and protocols. Factors contributing to these inconsistencies include variation in diet composition (larvae fed with plastic only or co-diets at different ratios that favor the enrichment of different taxa), experiment duration (causing a fluctuation in the presence and/or the relative abundance of gut microbes), larvae sources, (strongly affecting the gut microbiota composition). It is therefore desirable that the plastic diet administered to insects is standardized across studies since inconsistencies in the type, size, or composition of plastic used can lead to variations in results. Also, the insects' rearing conditions, including temperature, humidity, and other factors, can influence the gut microbiota and the rate of plastic degradation. The genetic variability behind insects used can be important to define their ability to degrade plastic in association with the gut microbiota.

Additionally, ensuring an adequate sample size and replication of experiments can enhance the reliability of findings. The duration of the experiment can affect the observed plastic degradation. Some studies may be short-term, while others may extend over a more extended period. Researchers should justify the chosen duration and consider potential long-term effects.

Despite the variety of experimental conditions applied, specific taxa recur in different studies as linked to the degradation of plastic, particularly Proteobacteria (specifically Gammaproteobacteria of the *Enterobacter* and *Pseudomonas* genera) and Firmicutes (including *Bacillus*, *Lactococcus*, and *Enterococcus* genera), demonstrating the presence of a core microbiota that operates the biodegradation. The introduction of standardized approaches to precisely delineate the distinct role of specific microorganisms within the gut microbiota would be desirable. However, we need to take into account microbic metabolic redundancy, i.e. different microbes may play the same role in the plastic degradation process, which may challenge the definition of the degradative role at the species level.

The elucidation of the degradation process carried out by the gut microorganisms would facilitate the development of super-degrading insects through specific gut microbiota modifications.

13.4. Biocontainment of the spillover of transgenic animals in the environment

The development of transgenic animals for waste management procedures, necessitates biocontainment protocols and strategies to prevent the escape of transgenes into natural populations. The scientific community must implement robust biocontainment mechanism to address public concerns, protect the environment from the uncontrolled proliferation of Genetically Modified Organism (GMOs). The unintended transgene escape (Ryffel, 2014) is a particularly relevant issue in genetically modified plants, where pollen can spread through the air and can be carried by insect or wind to distant locations uncontrollably.

Transgenic insects deployed in the free environment suffer from the same limitations and should be contained using specific strategies. Many of these strategies are also used to reduce the size of harmful populations (e.g., vectors of viruses and parasites representing a treat for human health), including sterile insect techniques (Bourtzis and Vreysen, 2021), sex-specific lethality (Zhao et al., 2020b), underdominance (Reeves et al., 2014), meiotic drive and sex ratio distortion systems (Compton and Tu, 2022).

The use of insects to process plastic wastes does not strictly require the release of insects in the environment, since they can be applied in confined spaces where the degradation process occurs. Consequently, less sophisticated biocontainment techniques can be adopted. Since a large collection of genes that impact the development of *D. melanogaster* are known, they can be easily implemented in containment strategies based on the development arrest at specific stages that do not interfere with the degradative abilities of the transgenic strain. As described above, larvae can be the stage of choice for insect-mediated plastic treatment. Since the subsequent developmental stages (pupae and adults) are less useful for this purpose, a possible strategy to avoid undesired dissemination of transgenes in the wild is to block the development of transgenic strains at the pupal stage. Several studies have reported the discovery of alleles associated to pupal lethality in *D. melanogaster* (Shearn, 1974; Perrimon et al., 1985; Ionascu and Ratiu, 2022). In addition, engineered conditional lethal mutations could be advantageously adopted to protect the environment from transgene contamination. The RIDL system (Release of Insects carrying a Dominant Lethal) uses the toxic overexpression of the tet-transactivator (tTA) in a self-promoting effector cassette, a lethal condition in the absence of tetracycline. This system is highly effective in several insect species (Gong et al., 2005; Harris et al., 2011; Schetelig and Handler, 2012) and could also be used to safely rear the transgenic flies expressing the plastic-degrading enzymes in the presence of tetracycline.

14. Concluding remarks

The above observations suggest that coupling the transgenic technologies with microbiome engineering could lead to the generation of economically tractable and environmentally sustainable plastic degraders. To maximize the potential benefits of employing transgenic insects in plastic waste management and recycling many more enzymes and genes should be characterized from plastic-degrading animals. Out of 249 organisms, only 62 enzymes and 34 genes have been characterized at least partially (Supplemental Tables 1 and 2). To overcome this limiting factor, fundamental research should play a pivotal role to perform the preliminary characterization in known and still unexplored living organisms, so that more combinations of transgenes can be expressed and tested to design an efficient plastic degrading transgenic model insect. Thanks to the advanced level of knowledge and technology achieved in the field *D. melanogaster* applications, still appears to be a promising organism in which plastic degradation characteristics can be integrated and modeled. Future advancement in genome annotation,

functional genetics, systems biology, and synthetic biology will offer more opportunities for the development of super-degrading insects. Furthermore, microbiota manipulation could be a way to enhance the plastic degradation capabilities of modified insects and requires to be thoroughly investigated to setup efficient bioreactors for plastic degradation and insect rearing.

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CRediT authorship contribution statement

Eugenia Pignataro: Data curation, Writing – original draft, Writing – review & editing. **Francesco Pini:** Data curation, Writing – original draft. **Alessandra Barbanente:** Data curation, Writing – original draft. **Fabio Arnesano:** Data curation, Writing – original draft. **Antonio Palazzo:** Data curation, Supervision, Writing – original draft. **René Massimiliano Marsano:** Conceptualization, Data curation, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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