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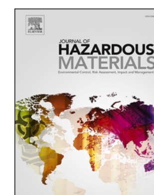

New insights on the degradation of polystyrene and polypropylene by larvae of the superworm *Zophobas atratus* and gut bacterial consortium enrichments obtained under different culture conditions

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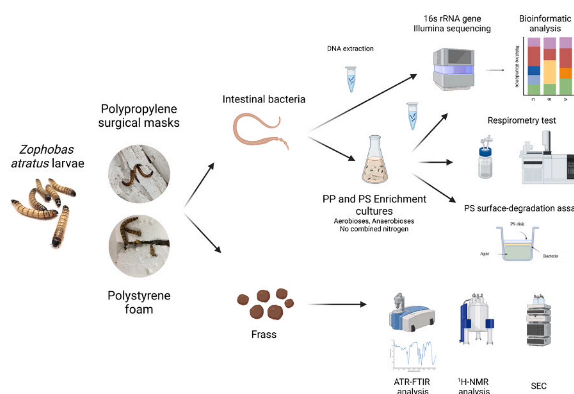
HIGHLIGHTS

- High survival rates are obtained for *Z. atratus* fed on polypropylene surgical masks.
- Proper cleaning treatments remove oxidation signs from ingested PP or PS.
- The plastic present in the frass only showed limited depolymerization.
- Anaerobic conditions favoured PP and PS degradation and the presence of nitrogen fixers in enrichment cultures.
- Polymer degradation routes in the anaerobic gut and aerobic conditions may differ.

GRAPHICAL ABSTRACT



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ABSTRACT

This study aims to deepen knowledge of the biodegradation of plastics, focusing on polypropylene (PP) fabric from surgical masks and polystyrene (PS) by larvae of *Zophobas atratus* as well as of specialized bacterial consortia from their gut, which were obtained in different enrichment conditions (aerobic, anaerobic, presence or absence of combined nitrogen). Plastics ingested by larvae obtained in Spain did not show any signs of oxidation but only limited depolymerization, preferably from the lowest molecular weight chains. Gut microbiota

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Surgical mask
Tenebrio molitor

composition changed as an effect of plastic feeding. Such differences were more evident in bacterial enrichment cultures, where the polymer type influenced the composition more than by culture conditions, with an increase in the presence of nitrogen-fixers in anaerobic conditions. PS and PP degradation by different enrichment cultures was confirmed under aerobic and anaerobic conditions by respirometry tests, with anaerobic conditions favouring a more active plastic degradation. In addition, exposure to selected bacterial consortia in aerobiosis induced limited surface oxidation of PS. This possibly indicates that different biochemical routes are being utilized in the anaerobic gut and in aerobic conditions to degrade the polymer.

1. Introduction

Due to its intrinsic properties, like cheapness, versatility and varied mechanical properties [1], plastic has played a fundamental role in everyday life, making it a virtually irreplaceable material in today's society. The need for a cheap and versatile material led in 2022 to a total production of 400.3 million tons of plastics worldwide, 44 % of which are materials destined for packaging [2]. Both in absolute terms and in the category of plastics used for packaging, polypropylene (PP) and polystyrene (PS) contributing to 18.9 % and 5.2 %, respectively, of global plastic production. Concerning PP, its end-of-life management gained more and more interest during the COVID-19 pandemic, due its wide use in the personal protective equipment (PPE). PP constitutes up to 72 % of the PPE used in the healthcare environment, leading to a sharp increase in production [3].

Even though they are thermoplastic polymers and therefore, their reprocessing does not pose any particular problems, recycled products do not attract much interest in the market [4], and, as with other types of plastics, the primary fate is to be piled up in landfills or incinerated [5]. In both cases, it can become a point of genuine concern, as in the former case, due to its permanence in the environment, it can go through fragmentation, being a source of microplastics, with several related consequences also for human health, such as inflammation, genotoxicity, and apoptosis [6]. At the same time, PS and PP can release hazardous pollutants contained in the polymer or generated in the combustion phase when incinerated [6]. Therefore, using classical plastic treatment methods does not guarantee an effective disposal method, and it is now widely believed that biodegradation may play a vital role. Interest continues to be aroused by the ability of lepidopteran and coleopteran insect larvae to survive on plastic-only diets without any specific development alterations, in which gut bacteria's key role in plastic degradation and assimilation has been established [7–9]. Specifically, the degradative capacity of larvae of some species of invertebrates toward different types of plastics has been analyzed, including the larvae of the greater wax moth *Galleria mellonella* [10–14], the yellow mealworm *Tenebrio molitor* [8,13,15–21] and the superworm *Zophobas atratus* [7,19,22–25].

Degradative capacities for each species have been studied mainly for polyethylene (PE) [10,16], and PS [8,12,22] and to a minor extent for PP [19,26]. Although most works have been done with *G. mellonella* and *T. molitor*, *Z. atratus* has demonstrated high degradation and survival abilities in plastic-only environments for PE and PS [15,19,23,24] making this species more attractive for degradation assessments. Very low survival rates have been reported for *Z. atratus* on PP foam in comparison to *T. molitor* [19], although recent reports have obtained higher survival rates [27,28]. The capacity of *T. molitor* to survive on PP microplastics or PP fabric from disposable surgical masks has been also demonstrated recently, even though the obtained survival rates were significantly lower than in the bran-fed controls [26,29]. For this reason, in this work, these parameters were assessed using PP fabric, derived from 3-layer surgical masks [30], and compared with the degradation of PS foam.

It is generally accepted that intestinal bacteria play a key role in plastic degradation by these insects since the suppression of intestinal microbiota by antibiotic supplementation strongly impaired the plastic degradation ability in most cases [8,15,19,22,24], which led to the conclusion that both oxidation and depolymerization of ingested plastic is not carried out by the larva but by the gut bacterial community. Nevertheless, recent evidence points to a dual role of larvae and gut microbiota in the degradation of plastics [14,28,31]. The analysis of the intestinal microbiota via next-generation sequencing (NGS) techniques and metabarcoding analysis corroborated the thesis of an important role of the gut microbiota in plastic degradation by revealing a strong shift in the microbiota correlated to the diet composition, favouring the proliferation of bacterial genus representing a minority in the standard bran diet and potentially correlated with a degradative capacity toward the polymers used as substrate.

In order to study the degradation activity by gut bacteria *ex-situ*, most authors have attempted to isolate and study pure cultures, which allowed the identification of several novel bacterial species directly isolated from the gut of the larvae acclimated to the plastic diet, observing a lower degradation effectiveness of pure cultures in comparison with the degradation rates within the larvae [22,23,31,32]. Nevertheless, it is highly probable that the complementary metabolic activity of a bacterial consortium is required to effectively degrade the plastic, which could also partially explain the higher degradation capacity of the worms. Indeed, although being a less explored strategy, selected bacterial consortia obtained from enrichment cultures, in which the plastic constitutes the only carbon source, have been demonstrated to be more efficient for plastic biodegradation than the microbiota directly obtained from acclimated *T. molitor* [8] and therefore deserve further investigation. In vitro enrichment has also been explored for *Z. atratus* gut microbiota [33], but information regarding the effect of culture conditions on plastic-degrading bacterial consortia remains limited. Oxygen and combined nitrogen are expected to be limiting factors for bacterial metabolism, and an increase in nitrogen fixation activity in the gut of plastic-degrading insects has been already reported [34], but these factors have not been considered for *ex-vivo* degradation experiments. In fact, the supplementation of the plastic diet with bran or beeswax has been demonstrated to enhance polymer degradation, probably by providing a source of combined nitrogen or other micronutrients [16,19,22,24]. Since combined nitrogen availability is expected to limit the growth of the plastic-degrading bacterial consortium, we also hypothesized that the anaerobic conditions that favour nitrogen fixation might also favour the degradative activity in the isolated bacterial consortia. Therefore, and even though oxygen availability has been described to be essential for rapid plastic biodegradation [8,16], it would be interesting to investigate if anaerobic conditions that favour nitrogen fixation could help in *ex-vivo* degradation by enrichment cultures derived from the larvae gut microbiota or drive to the selection of different bacterial species/guilds.

Thus, this study aims to deepen our knowledge regarding the biodegradation of plastics, particularly PP fabric from surgical masks and PS, by larvae of *Z. atratus,* also known as *Z. morio* [35]. The capacity of PP degradation was compared with that of larvae of *T. molitor*. Survival under plastic-only diet conditions was evaluated for 28 days, assessing average weight and plastic consumption parameters. Novel *Z. atratus* survival capacity was observed when fed with PP fabric, and the ability to survive when fed with PS foam was confirmed. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy and size exclusion chromatography (SEC) were performed to confirm the presence of PS or PP in egested frass and assess their degradation.

The in vitro degradation capacity of gut bacteria was assessed by generating specialized bacterial consortia obtained in different enrichment conditions (aerobic, anaerobic, presence or absence of combined nitrogen) to observe whether they could lead to different degradative capacities and to compare the specialization of different consortia The consortia's degradative capacity was also assessed by a respirometry test, evaluating produced CO_2 by gas-chromatography (GC) and bacterial community specialization was followed by metabarcoding analysis, revealing the presence of bacterial families in the specialized consortium, not previously reported in the literature. Finally, the *ex-situ* degradation capabilities of the bacterial enrichment consortiums obtained from the *Z. atratus* gutswere evaluated through a new liquid-solid interface method designed to allow observation of surface oxidation of PS by ATR-FTIR spectroscopy.

2. Materials and methods

2.1. Source of larvae and test materials

Larvae of the species *Z. atratus* and *T. molitor* were purchased from an insect farm (Bichosa, Vigo, Spain) that fed them with bran. To prevent gut contamination from the previous diet, the larvae went through 48 h of starvation before being weighed and distributed among the different diets. Three conditions were set up with different feeding conditions, including control cultures maintained with bran, standard PS foam and PP fabric from 3-layer white surgical masks (Mask4u PI Medical labs, Badajoz, Spain) to investigate whether the low survival rates previously observed for PP foam could be improved by using fabric. The 3 layers of the surgical masks were used in the feeding experiment. Chemicals used in this project, including tetrahydrofuran (THF, GPC grade purity ≥99.9 %), deuterated chloroform ($CDCl_3$, NMR grade purity ≥99.8 %), and all of those not directly listed were purchased from Merck (Darmstadt, Germany).

2.2. Larvae rearing and plastic consumption

Larvae were reared in food-grade PP containers with an approximate volume of 1.8 L (19 ×12.5 ×7.5 cm), provided with a cover previously perforated to allow sufficient air inlet, containing 80 larvae *Z. atratus* or 220 larvae of *T. molitor* each. Larvae were cleaned with a compressed air stream and weighted before being distributed in the containers. The average initial weight of the larvae ranged from 0.65 to 0.70 g for *Z. atratus* and 0.11 to 0.13 g for *T. molitor*. To assess plastic consumption, four 10 cm squares of 1 cm thick PS foam, with a total weight ranging from 4.2–4.5 g (PS-fed condition), or a 3-layer PP mask, cut at the edges and deprived of the elastics were added to each container (1.3–1.5 g per container, PP-fed condition) All 3 layers were used since the chemical composition was found to be identical, but showed different texture, the external layers were made of woven-not-woven PP meanwhile the internal PP melt-blown layer was thicker and softer. Control cultures were maintained with an excess of bran. The control and PP-fed conditions were also evaluated for *T. molitor* for comparison purposes. Three replicates were set for each condition. The feeding period lasted 28 days, during which the larvae were kept at constant humidity (75 % ± 5) and temperature (25ºC) in complete darkness. Every 2 days, the larvae were counted, and dead larvae were removed to avoid cannibalism. Weekly, the survival rate and the weight variation were measured. Percentage of weight loss and specific PS/PP consumption rates (SPCR) were determined weekly as milligrams of PS or PP consumed by 100 larvae per day (mg PP·100 larvae^{-1} d^{-1} and mg PP g larvae^{-1} d^{-1}) [24].

An additional experiment was conducted with *Z. atratus* to assess the role of gut microbiota in PP degradation. Larvae were fed with bran treated with gentamicin (30 mg per gram of bran [15,19,22,24] for 5 days and transferred to clean containers without food for 24 h before being transferred to containers with PP surgical masks (average initial weight 2.5 g). Non-treated larvae were set as controls. Larvae (n = 30) were distributed in containers in triplicate and maintained for 21 days. Gentamicin (60 mg) was added to the antibiotic-treated containers on days 0 and 11. Survival was recorded every 2 days, and dead larvae were removed. The gut microbiota of the larvae was retrieved for DNA extraction and metabarcoding analysis at the experiment's beginning and end, as explained below. Additionally, at the end of the experiment, another 10 larvae from each control and antibiotic-treated container were cleaned softly with compressed air and transferred to clean Petri dishes for 24 h to harvest the frass, which was analyzed for bacterial composition.

2.3. Polymer characterization in the frass

At the end of the 28-day test, the *Z. atratus* larvae were gently cleansed with compressed air and transferred to a clean container for 48 h for frass collection. ATR-FTIR spectra of the reference PS and PP, and frass samples from control, PS-fed, and PP-fed larvae were obtained using a Spectrum Two FTIR Spectrometer (PerkinElmer, Shelton, Connecticut, USA) equipped with diamond internal reflection element from GladiATR accessory S2PE (PIKE Technologies Inc, Cottonwood Dr. Madison, Wisconsin, USA) to detect possible changes in the major functional groups. The spectra of all samples were obtained in transmittance mode, in the spectral region of 400–4000 cm^{-1}, using a resolution of 4 cm^{-1} and with 32 scans. To remove small water-soluble molecules from frass, approximately 10 mg samples were washed three times with 5 mL of Milli-Q water and dried for 12 h in an under vacuum oven at 40 °C.

^{1}H NMR analysis using a Bruker DRX-500 (Billerica, Massachusetts, USA) was also performed to follow the formation of functional groups or changes in composition in frass samples from PS-fed larvae. At room temperature, 30 mg of frass samples was extracted under vigorous stirring in 1 mL of THF for 24 h. The solution was filtered through a 0.22 μm PVDF membrane to remove all the non-dissolved components and let evaporate until complete drying. Finally, the solid was dispersed in 0.8 mL of $CDCl_3$ for the analysis. Diffusion filter experiments (^{1}H Dfilter) were also acquired to remove or strongly attenuate solvent signals and other low molecular weight molecules. Dfilter spectra were acquired using a BPP-STE (Bipolar Gradients Stimulated Echo) experiment. The diffusion delay time was 90 ms. The diffusion encoding gradients have a sinusoidal shape, a duration of 2 ms and a power level of 29 G cm^{-1}.

SEC measurements were carried out using a PL-GPC 50 GPC System (Agilent Technology, Santa Clara, California, USA) apparatus equipped with a MIXED-E column (5 μm, 7.5 mm × 300 mm) with a nominal exclusion limit of 2000,000 Da, to determine the variation of number average and weight average molecular weight (M_n and M_w, respectively) between the virgin, reference, PS and the residual PS in the frass of the PS-fed group. A refractive index detector was used, and column calibration was performed with PS narrow distribution standards. A third-order polynomial equation was obtained from the regression analysis. 200 mg of frass samples were extracted in 5 mL of THF for 24 h at room temperature under vigorous stirring. The extracted solvent was filtered through a 0.22 μm PVDF membrane and dried at room temperature. Then, the obtained extract was dissolved in 3 mL of THF. In the case of reference PS, 200 mg of sample were directly dissolved in 3 mL of THF.

2.4. Recovery of gut bacteria and DNA analysis

At the end of the feeding period, 13 *Z. atratus* larvae for the control condition and 28 for both PS- and PP-fed conditions were retrieved, placed in a 70 % ethanol bath until completely anaesthetized and then dissected with sterile instruments by removing the head and the tail, making the guts easily removable. Guts from control and plastic-fed groups were resuspended in 10 and 15 mL respectively of liquid carbon-free basal medium (LCFBM) saline solution (pH: 6.51, 0.7 g of KH_2PO_4, 0.7 g of $MgSO_4$·7 H_2O, 1.0 g of NH_4NO_3, 0.005 g of NaCl, 0.002 g of $FeSO_4$·7 H_2O, 0.002 g of $ZnSO_4$·7 H_2O, and 0.001 g of $MnSO_4$·H_2O in

1 L of deionized water) [23] and homogenized for 15 min in a Stomacher lab blender 80 (Seward, Worthing, UK). The same procedure was applied to the larvae in the antibiotic treatment experiment. In this case, 5 larvae were analyzed for each replicate and gut content was resuspended in 7,5 mL of PBS. In this experiment, the number of colony forming units (CFU) was assessed by serially diluting the gut suspensions in PBS and plating on LB agar. Plates were incubated at 20ºC for 10 days, and colonies were counted on plates presenting colonies in the range 30–300.

For bacterial DNA analysis, 5 mL samples of the gut homogenates were centrifuged and washed 3 times with LCFBM saline solution. The remaining pellets and the frass harvested in the antibiotic-treatment experiment were extracted using the DNeasy PowerBiofilm Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The resulting DNA was quantified and quality assessed before the amplification of the V3-V4 hypervariable region using Bakt_341F (5′ CCT ACG GGN GGC WGC AG 3′) and Bakt_805R (5′ GAC TAC HVG GGT ATC TAA TCC 3′) [36]. DNA metabarcoding analyses were carried out by AllGenetics & Biology SL (www.allgenetics.eu) using Illumina® sequencing technology. Bioinformatic analysis was performed using the Microbial Genomics module (version 21.1) workflow of the CLC Genomics workbench (version 21.0.3). Briefly, raw sequences were filtered to remove low-quality reads and then clustered into Operational Taxonomic Units (OTUs) at 97 % cutoff for sequence similarity and classified against the non-redundant version of the SILVA SSU reference taxonomy database (release 132; http://www.arb-silva.de). Further assays were performed on the most abundant bacterial OTUs (above 0.5 % of the observed OTUs).

2.5. Consortia enrichment

The gut bacteria suspensions obtained, as explained above, were used as inoculum for enrichment cultures to eventually select consortia specialized in plastic degradation for use in other phases of the work. For each condition, 5 mL of the intestine's homogenates were recovered after sedimentation of large debris and transferred into a 50 mL Erlenmeyer flask previously sterilized by autoclaving and containing 25 mL of sterilized LCFBM saline solution and 100 mg of PP or PS previously ground with a high-speed multifunctional grinder, and disinfected with ethanol 70 %. Since the PP layers showed the same composition, but it was not feasible to grind the external ones, only the internal melt-blown layer was used in this step. The enrichment cultures were maintained in aerobiosis (PS AE and PP AE) and anaerobiosis (PS ANA and PP ANA). Additionally, the consortium's growth in the absence of combined nitrogen was tested in anaerobiosis (PS ANA-N, and PP ANA-N) using LCFBM medium without NH_4NO_3. Cultures were maintained at 20ºC in a shaker at 120 rpm. Anaerobic cultures were maintained in an anaerobic jar with AnaeroGen™ 2.5 L (Thermo Fisher Scientific corporation, Waltham, Massachusetts, USA). After 5 days, a 5 mL aliquot from each flask was transferred to a new flask containing fresh saline solution and the same amount of ground disinfected plastic. The process was repeated after one week and then every 15 days, with a total enrichment period of 2 months and 5 enrichment passages.

2.6. Ex-situ degradation of PS

The plastic degradation capacity of specialized consortia was evaluated using a new method specially developed for the experiment. The degradation was performed in 12-wells cell-culture plates (Falcon Polystyrene Microplates, Corning, New York, USA), filled with 3 mL of a LCFBM solution containing 15 g L^{-1} agar. Then 100 μL of the resuspension containing the consortia were inoculated on the solidified LCFBM agar, and finally, a sterilized PS disc (0.8 cm diameter) was placed on top of the bacterial drop. Conditions of aerobiosis or anaerobiosis were maintained accordingly, depending on the consortium's prevenience. All cultures were performed in triplicate. Disk degradation was assessed by FTIR spectroscopy after 14 and 28 days, after being subjected to a special five-step cleaning protocol specially developed for this purpose (discussed in the Supplementary Material, including Fig. S1). Firstly, with the help of sterilized forceps, the disks were placed in sterile 15 mL tubes and subjected to a first cleaning step by adding 2–3 mL of Milli-Q water, letting them soak, and subsequently performing ultra-sonication in a warm (40–45 °C) water bath for 15 min. In a second step, water was removed from the tubes and 5 mL of a 5 % solution of sodium dodecyl sulfate (SDS) was added, and tubes were submitted to a second 15 min cycle of sonication to remove any attached organic molecules. Disks were washed 3 times with 5 mL of Milli-Q and shaken vigorously. Finally, the disk is rinsed in 2–3 mL of Milli-Q water, ultra-sonicated for 5 min in a warm water bath, and dried at 40 °C under vacuum for 4 h. A detailed description of the optimization of the disk cleaning procedure is provided in the SI. ATR-FTIR spectra of the cleaned disks were acquired with a 4 cm^{-1} resolution and 32 scans. In addition, exploratory ^1H NMR analysis were carried out on PS powders obtained by scraping the cleaned disk surface with a scalpel.

Bacterial proliferation onto PS disks was observed before the cleaning procedure with scanning electron microscopy (SEM) Zeiss FESEM Ultra Plus (Oberkochen, Germany). Confocal Laser scanning microscopy (CLSM) Leica TCS-SP2 (Wetzlar, Germany) was also used to assess better the distribution of the bacteria on the plastic surface, which was stained with the Invitrogen LIVE/DEAD BacLight Bacterial Viability Kit Protocol (Thermo Fisher Scientific corporation, Waltham, Massachusetts, USA).

2.7. Respirometry test

This test was performed to evaluate the degradation capacity of the different bacterial consortia. Watertight 50 mL bottles equipped with a perforable septum cap were filled with 25 mL of LCFBM with or without nitrogen and 100 mg of ground and disinfected PP or PS and were inoculated with 5 mL of the bacterial enrichments obtained after two months. The source of PP and PS was the same as used in the consortium selection experiments. All conditions were assayed in triplicate. Two control bottles were set for each polymer type, one non-inoculated bottle containing only LCFBM saline solution and the polymer and the other containing only 5 mL of the bacterial consortium and nitrogen-free LCFBM saline solution without plastic. Bottles were incubated in aerobiosis and anaerobiosis. In this case, the anaerobic condition was created by flushing an excess of pure nitrogen into the bottles. Bottles were maintained in constant agitation at 120 rpm at 25 °C. To evaluate CO_2 production, the composition of the gaseous phase of the bottles was analyzed every week for 28 days by GC. 1 mL of gas was removed from the bottle with a 1 mL GC syringe and injected in a Hewlett Packard 5890 Series II (Palo Alto, California, USA) equipped with a Supelco Porapack Q 80/100 2 m x 1/8" column (Sigma Aldrich, Burlington, Massachusetts, USA) and a thermal conductivity detector. A gas of known concentration was used for the calibration so that the area subtended in the chromatograms of the samples could be correlated with the molar per cent concentration. At the end of the 28-day incubation period, PS was analyzed by performing SEC analysis with PL-GPC 50 GPC System (Agilent Technology, Santa Clara, California, USA). PS was filtered with 100 and 10 μm sieves, sonicated with a 5 % SDS solution and washed with Milli-Q water to remove biomolecule contamination. Cleaned PS samples were dried in a vacuum oven for 24 h at 40 °C and finally dissolved in THF for measurement.

3. Results and discussion

3.1. Survival and growth of Z. atratus on PS and PP

The ability of *Z. atratus* to survive on PP and PS was assessed by larvae and pupae counting and plastic consumption following previous investigations [16,19,24]. In the present work, the highest survival rate (SR) after 28 days was obtained with PP from surgical masks (86.67 ±

7.64 %), being slightly higher than the in the control group fed with bran (79.17 ± 8.32 %), with the lowest survival rate being observed in the PS-fed group (76.67 % ± 2.60 %) (Fig. 1), even though these differences were not statistically significant (T-test, $p < 0.01$). The SR obtained here with PP fabric is much higher than that reported for PP foam (12 %) by other authors [19] and similar to the SR reported recently with microplastics [28] or with PP foam of lower molecular weight [27]. These differences could be caused by the difference in the surface of the PP material available for enzyme degradation or the lower density and higher porosity of the material. During this assessment, a preference for consuming the melt-blown inner layer of the masks rather than the outer layers could be observed, a fact that has been also observed in *T. molitor* [29]. Although the chemical composition of the layers was identical, the texture was significantly different. The softer inner layer was attacked first, and only at the end of the incubation period, the larvae attacked the thinner outer layers (Fig. S2). Differences in survival and degradation capacity of PS foam have been reported for different *Z. atratus* strains [24], which could be derived from the initial microbial endowment of the larvae. Values close to 100 % survival have been reported for PS-fed *Z. atratus* [14,24,25] while other authors report SR around 70 %, similar to those reported here after a similar period [22]. Again, these differences could also be attributed to differences in degradation capability between *Z. atratus* strains or different characteristics in the PS used as feed.

In a previous work, the survival of *Z. atratus* on PP foam was reported to be very low, 12.1 %, compared with the yellow mealworm larvae (*Tenebrio molitor*), 88.7 %, despite the high PP consumption reported [19]. In *Z. atratus*, the SR greatly improved by supplementing PP foam with bran, while the supplementation did not affect *T. molitor* [19]. For this reason, the survival and consumption of both species were also assessed here, using PP fabric from surgical masks instead of PP foam. The SR observed in *T. molitor* cultures that were maintained in parallel for 21 days on PP fabric (69.4 %) were lower than the controls maintained with bran (84.7 %) (Fig. S3) and also lower than the SR observed in *Z. atratus* in the same period (88.33 %) (Fig. 1). A lower SR also was reported for *T. molitor* fed with different layers of PP surgical masks [29] or PP microplastics [26] in comparison with the bran diet. In addition, the performance of *Z. atratus* larvae in terms of survival and polymer consumption has already been shown to be superior to *T. molitor* and *G. mellonella* larvae reared on PS [13], indicating a general superior capacity of the superworm for adapting to different diets. Very low pupation rates were observed in *Z. atratus* after 28 days (1.7 % in PS-fed, 2.1 % in PP-fed) in comparison with *T. molitor*, and, surprisingly, pupae were only observed in the PP and PS-fed cultures after day 21, but not in bran-fed cultures (data not shown). These low pupation rates were expected since, in general, two months are required in *Z. atratus* to achieve the pupae stage when fed PS [22]. Notably, the percentage of pupae observed in *T. molitor* fed with PP fabric was very high, reaching values of 60 % of surviving animals (Fig. S3b). This value is much higher than those reported for *T. molitor* on different plastic materials, which remained below 5 % [14,17], confirming its ability to subsist on a PP fabric diet.

Regarding the average weight of *Z. atratus* larvae (Fig. 1b), a slightly negative trend was observed for both plastic-feeding conditions, even though a decrease in average weight was also observed in the bran-fed group. This phenomenon led to a weight loss of 5 % in the control group, 3.1 % in the PS-fed group, and 9.7 in the PP-fed one, with no significant statistical differences among treatments (T-test, $p < 0.01$). Similar weight losses have been reported for different polymers [14,22,25,27]. However, despite high survival, a much higher weight loss

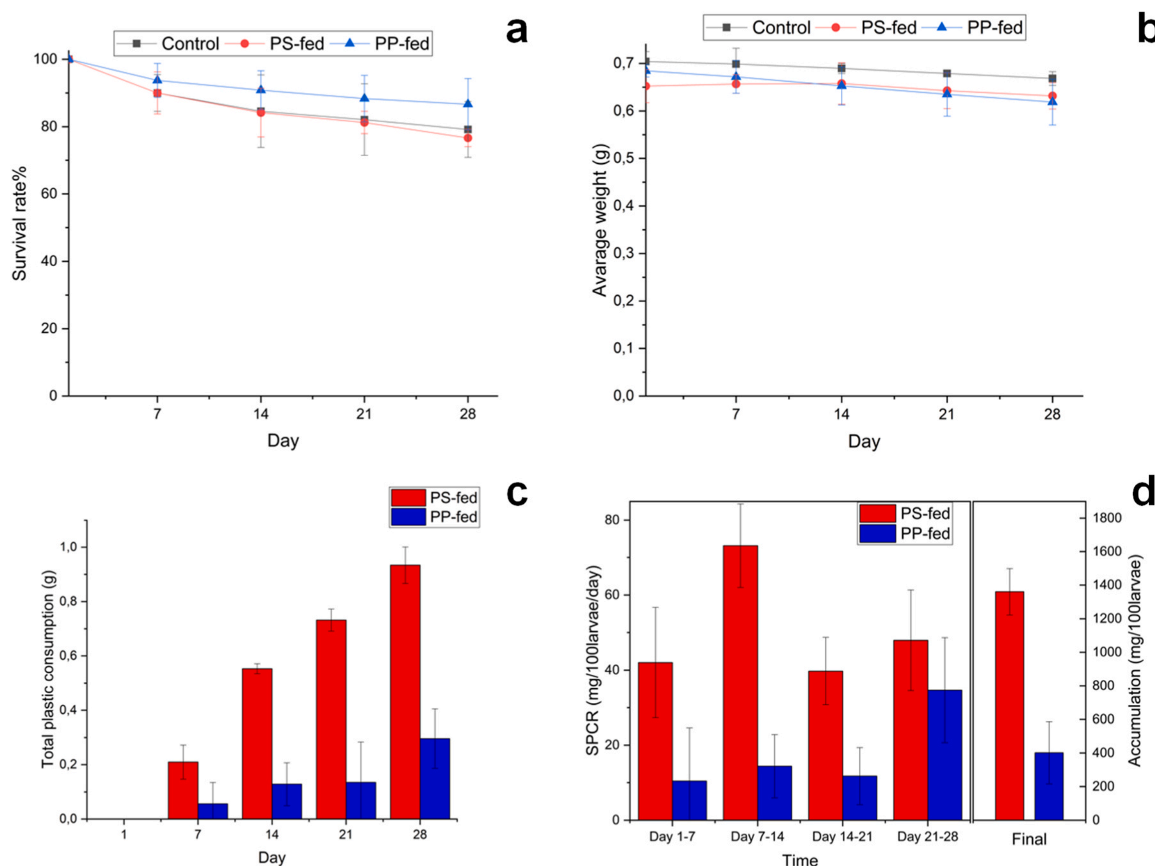

Fig. 1. Z. atratus larvae survival rate (a), average weight (b), total plastic consumption per container (n = 80 larvae) (c), and specific plastic consumption rate (SPCR) (d), maintained for 28 days on PS (PS-fed, red symbols and bars) and PP (PP-fed, blue symbols and bars). Survival and average weight of control cultures maintained with bran are also shown. Vertical bars represent the average of 3 replicates.

(−55.6 %) was reported for a PS diet in *Z. atratus* in a similar cultivation period [13]. On the contrary, discrete weight gains (+5.2 %) have been reported for PS diets, which improved with bran supplementation [24]. As for survival, weight gain/loss is strain-dependent [24]. While weight loss has also been reported for *Tenebrio* sp. fed different plastic materials [13,18], in our experiments, the weight of *T. molitor* larvae fed on PP fabric remained mostly unchanged (Fig. S3c). When PP is presented as low molecular weight microplastics, a significant weight gain has been reported for *T. molitor* [26], indicating the importance of the mode of presentation and characteristics of the polymer for digestibility.

Concerning plastic consumption, total plastic consumption and specific plastic consumption rates (SPCR) were evaluated. It was observed that *Z. atratus* could consume, on average, 0.93 ± 0.07 g of PS and 0.30 ± 0.11 g of PP per container (initial larvae number =80) at the end of the 28-day experiment (Fig. 1c). Despite the ingestion of plastic was lower with PP fabric, larvae survival was higher than with the PS, while larvae weight loss was similar with both diets (Fig. 1a,b, and Fig. 3a,c). That may indicate that larvae are eating a higher amount of PS due to a lower digestibility. In *Z. atratus*, the SPCR ranged between 40 and 70 mg·100 larvae^{-1} d^{-1} for PS and 10–35 mg·100 larvae^{-1} d^{-1} for PP (Fig. 1d). The values obtained for the PS group align with the highest values reported in the literature for PS consumption, which range from 15.5 ± 8.7 to 77.0 ± 2.5 mg·100 larvae^{-1} d^{-1}, depending on the strain of the larvae and if the diet includes only PS or is combined with bran

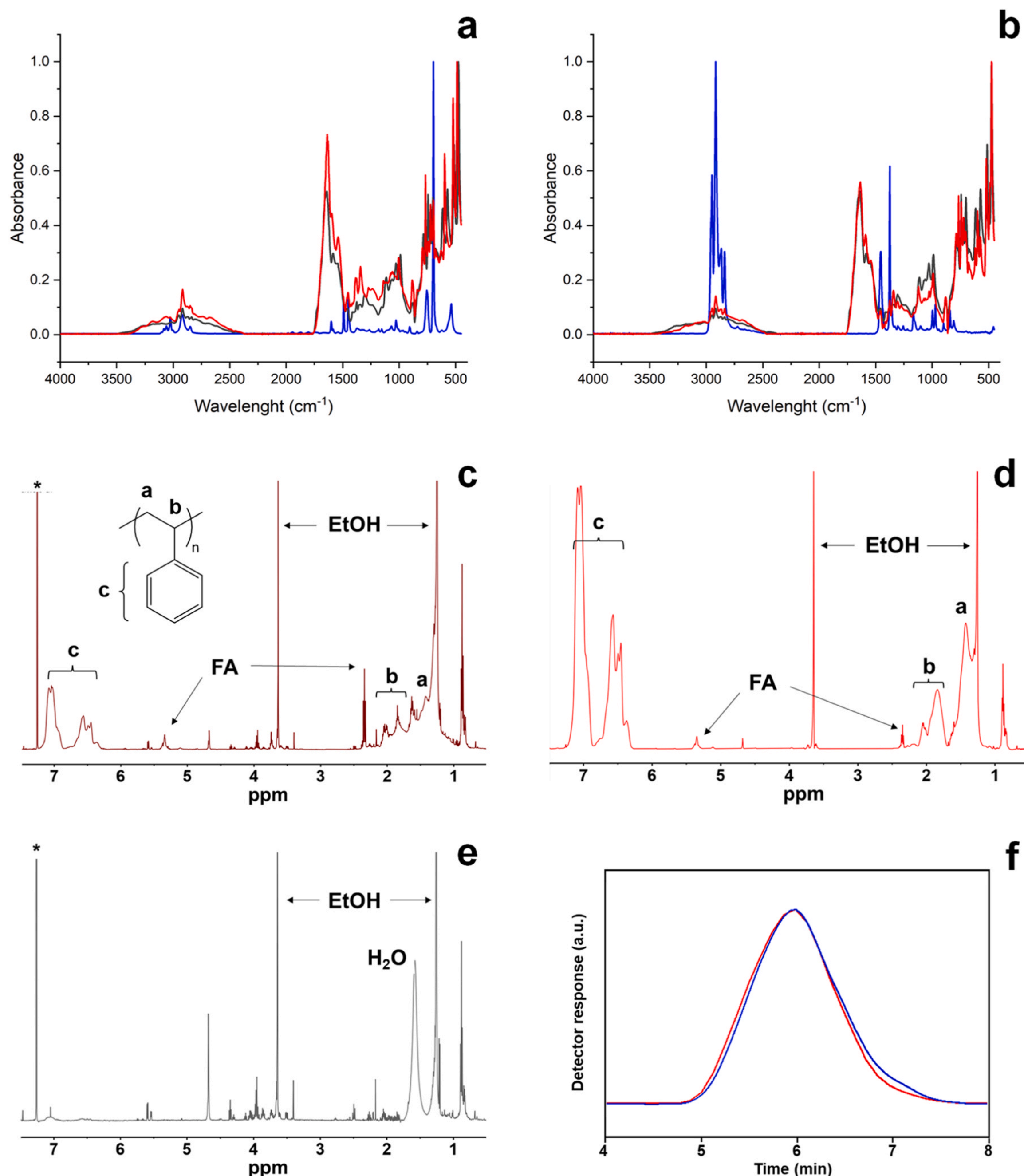

Fig. 2. ATR-FTIR spectra of: a) reference PS (blue), Z. atratus control group frass (grey) and PS-fed group frass (red); b) reference PP (blue), Z. atratus control group frass (grey) and PP-fed group frass (red). NMR spectra of PS-fed group frass, before (1D ^{1}H, c) and after the application of a diffusion filter (^{1}H Dfilter, d), and control group frass (1D ^{1}H, e); signals a-c refer to different PS protons; FA denotes peaks ascribed to fatty acids; asterisk mark denotes signal due to undeuterated $CDCl_3$. Normalized SEC curves (f) of reference PS (blue) and PS-fed group frass (red).

[24]. On the contrary, the SPCR reported here for PP is much higher than the values reported in the literature, 3.1 ± 0.4 mg·100 larvae^{-1} d^{-1} [19]. This difference can also be related to the different PP properties, as a PP melt-blown and non-woven fabric was used in this work to feed larvae, instead of a very low-density PP foam.

As expected, due to the lower weight of *T. molitor* larvae, the final SPCR obtained for PP with *T. molitor* after 21 days, 22.5 mg·100 larvae^{-1} d^{-1} (Fig. S3d) is much lower than with *Z. atratus* 401.2 mg·100 larvae^{-1} d^{-1} (Fig. 1d). In order to be able to compare the ingestion rates of PP obtained with *Z. atratus* with those observed in *T. molitor* larvae, weight-normalized SPCR (SPCR-W) was calculated, confirming a superior ingestion of *Z. atratus*, 6.1 mg·g larvae^{-1} in comparison with *T. molitor* 1.96 mg·g larvae^{-1}, corroborating the previous results reported for PS [13,30]. On the contrary, a much higher SPCR-W of PP has been reported for *T. molitor* in comparison with *Z. atratus* [19].

3.2. Frass analysis

Different techniques were applied to follow polymer degradation in the frass, including ATR-FTIR and NMR spectroscopies, and SEC. FTIR spectroscopy was widely used to characterize, and study egested frass from larvae to follow polymer structural changes [12,24]. In particular, the appearance of oxygen-containing groups has been reported for PE, PP and PS, corresponding to the observation of new peaks in the FTIR spectra associated with hydroxyls (OH stretching) at 3500 cm^{-1}, carbonyl groups at 1700 cm^{-1}, formation of new double bonds at 1650 cm^{-1}, and insertion of oxygen in the polymeric chain (C-O-C stretching) at 1075–1150 cm^{-1} [17,18,24].

The ATR-FTIR spectrum of the frass of the *Z. atratus* PS-fed group, compared in Fig. 2a with that of the reference PS used for feeding, reveals the presence of peaks, like those due to C-H stretching at 2900–3000 cm^{-1}, CH_2 bending at 1450 cm^{-1} and aromatic CH out-of-plane bend at 690 cm^{-1} [37], which may be related to the presence of the polymer. In addition, remarkable differences are visible, such as an extensive broadening and appearance in the frass of new peaks in the 2500–3500 cm^{-1} range, a strong peak at around 1700 cm^{-1}, and the general increase of absorptions and appearance of new peaks between 900 and 1400 cm^{-1}. All of these changes could be associated with hydroxyl groups, carbonyl groups, and other oxygen-containing groups, which presence in the polymer chain was proposed as the preliminary and key step in plastic degradation since it would allow the transition of a hydrophobic surface toward one with more hydrophilic properties [18].

However, a comparison with the spectrum of the frass of the control group fed with bran, also shown in Fig. 2a, is considered essential to evaluate the structural features correctly. As a fact, no clear differences between the frass deriving from the bran-fed control and those from PS-eating groups can be pointed out. The 2500–3500 cm^{-1} region and the strong peak at 1650–1700 cm^{-1} are very similar in the two spectra, meaning that these signals are due to some compounds present naturally in the frass, as molecules or macromolecules produced in the gut during the digestion process, and are not associated with any oxidation of the polymer. Only the C-O-C stretching region shows some differences, which may be related to either some process of oxygen insertion in the polymer chain or different metabolic molecules due to the different diet.

Therefore, the formation of new functional groups in the ingested PS cannot be confirmed by FTIR spectroscopy as in previous investigations [13,19,24,25]. A similar assessment can be conducted for the PP-fed group (Fig. 2b). Like in the previous case, all the absorptions due to hydroxyl and carbonyl groups and to C-O-C stretching, eventually proving PP degradation, are very similar in the spectra of the frass of both the control and the PP-fed groups. Although these results do not exclude polymer degradation, they corroborate the well-known low sensitivity of FTIR spectroscopy, especially limited when the spectral bands of the targeted functional groups overlap with those of other major components. Unless some selective frass extraction or cleaning process is applied, as shown below for the PS degradation by isolated single bacteria culture, ATR-FTIR spectroscopy is ineffective in detecting polymer biodegradation in frass, suggesting the need to use a more sensitive analytical techniques, such as NMR.

The ^{1}H NMR spectra of THF soluble fractions of frass from PS-fed and bran-fed groups are compared in Figs. 2c and 2e. In addition, a diffusion filter with presaturation was applied to the PS-fed group frass to reduce signals of components with large diffusion coefficients, such as solvents and small metabolic molecules (Fig. 2d). In such a way, PS signals, having a smaller diffusion coefficient, are affected to a much lesser level and are easier to read.

The typical main peaks due to PS protons and the large variety of signals associated with the standard metabolic activity of gut microbes and enzymes of *Z. atratus* are discernible in the frass of the PS-fed groups (Fig. 2c). Only a few additional peaks are visible in the spectrum of PS-frass, namely a multiplet centred at 2.30 ppm and a triplet at 5.35 ppm, which were previously ascribed to the formation of new alkene bond, -CH=CH-, and hydroxyl group, -OH [19,24]. In addition, the fact that very similar signals were also detected in the frass of *Z. atratus* fed with diverse polymers, such as PP and PE [16,19,24], where the formation of the same type of groups would give chemical shifts influenced by the peculiar neighboring groups of each polymer (e.g. methyl groups in PP vs. aromatic rings in PS), do not support such hypothesis. Only a recent work by He et al. [26] on PP microplastic biodegradation by *T. molitor* showed an additional peaks around 3.6 ppm due to aldehyde moiety, possibly indicating oxidation and degradation.

The application of diffusion filtering to NMR measurements [38] revealed the low molecular weight nature, among the others, of the substances associated with the peaks centred at 2.30 ppm and 5.35 ppm. On the other hand, both peaks perfectly fit with the presence of fatty acids in the egested frass of polymer-fed *Z. atratus* [13], being the α-CH_2 to the carbonyl group of the acids and the typical olefinic hydrogens of unsaturated fatty acids, the groups responsible of the peaks at 2.30 ppm and 5.35 ppm, respectively. All other fatty acid peaks overlap with those corresponding to substances present in both bran-fed and PS-fed frass and cannot be univocally identified. The source of these unsaturated fatty acids could be the membranes of the bacteria present in the intestine of the larvae or the short-chain fatty acids derived from the fermentative metabolism of bacteria, which may even constitute the main nutritional source for the larvae [39,40]. Finally, it is worth mentioning that the presence of ethanol (EtOH) in all the spectra may be due to microbial fermentation.

As structural changes in the polymer as an effect of ingestion appear negligible, or at least at levels lower than the limit of the applied spectroscopic techniques, a common practice consisting of evaluating molecular changes by SEC measurements was applied to prove and follow polymer degradation. Although a wide range of solvents may be used to dissolve different polymers, in this work, the determination of molecular weights and their distribution was limited to PS, which is easily soluble at room temperature in a common SEC solvent such as THF. In contrast, the study of PP would have required high-temperature chromatographic systems, intrinsically more complex and less sensitive than standard equipment to the limited molecular weight changes expected [24]. Molecular weight distribution of the frass of the *Z. atratus* PS-fed group is compared in Fig. 2f with that of the PS used for feeding, while the number average molecular weight, M_n, and the weight average molecular weight, M_w, were 92,400 and 100,300 Da, and 240,700 and 227,500 Da, respectively. The polydispersity index, DPI, decreased from 2.61 to 2.55. The molecular weights of the THF-soluble fractions of the frass of the bran-feed control group (results not shown, corresponding to < 5 wt% of the frass amount) were in the range 300–8,000 Da, with M_n = 500 Da. The presence of these very low molecular fractions in the frass of the PS-fed groups is negligible with respect to the polymeric fraction and is not discussed hereafter.

The increase in the value of M_n and the decrease in M_w indicates a general process of limited depolymerization, as the larvae obtained from

a USA source but differently from those obtained from a Chinese source [24]. On the other hand, the almost complete overlapping of the molecular weight distribution of PS before and after ingestion, except for the complete disappearance of the low molecular weight fraction of the polymer up to around 10,000 Da, reveals more details on the mechanism of biodeterioration. The reduction in M_w as an effect of degradation demonstrates the occurrence of polymer chain cleavage. At the same time, the change of M_n indicates the increase in the average length of the remaining polymer chains. These findings support the hypothesis that shorter chains are more easily degraded. We hypothesize a metabolization mechanism in which enzymatic sites have a higher affinity for lower molecular weight chains than for longer chains. If this type of selection by the enzyme was not present, a different degradation pattern would result, showing a more widespread stochastic breakdown, producing a clear M_n and M_w decrease, as well as a change in the molecular weight distribution and a dramatic increase in the DPI. In addition, it is possible to speculate that the metabolization mechanism does not proceed from the chain terminals by a mechanism comparable to unzipping. However, it preferentially occurs by a random cleavage mechanism of C-C bonds, preferably from the lowest molecular weight chains or the final ends having higher molecular mobility, making it difficult for enzymes to act if the polymer is not available in a form accessible to it.

3.3. Degradation capacity of bacterial enrichment cultures

A respirometry test was used to assess the degradation capacity of enrichment cultures obtained by successive transfers of *Z. atratus* gut microbiota into culture media containing only PP or PS as a carbon source [41]. The assays were performed in 50 mL bottles containing 25 mL of saline solution and 100 mg of grounded polymer, and degradation was monitored by measuring CO_2 production. For PP, only the inner melt-blown layer was used in the respirometry test, since this was preferentially attacked by the larvae and it was not possible to obtain a fine powder from the outer layers. The consortia had been acclimated to using PP or PS as the only carbon source for 2 months (5 passages) in aerobiosis and anaerobiosis. Additionally, with the hypothesis that N-fixing gut bacteria may represent an important source of protein for the larvae and other bacteria in the consortium [42], and therefore, significantly contribute to the degradation, the effect of the presence/absence of a combined nitrogen source was tested in anaerobioses. All the enrichment cultures produced a clear increase in CO_2 concentrations after 7 days (Fig. 3) for the two polymers compared to the uninoculated controls. The amount of CO_2 produced in the control cultures inoculated with the consortiums without plastics was similar to the uninoculated cultures with plastics (for simplicity, only the CO_2 evolution of uninoculated PP control flasks maintained in aerobiosis and anaerobiosis is shown in Fig. 3a). This trend testifies to the actual production of CO_2 by the bacteria, which should be related to plastic degradation, as the values recorded greatly exceed those obtained in the controls. This CO_2, excluding the percentages deriving from the air's composition in non-degradation (absence of bacteria) and that emanating from cannibalism, can only derive from the assimilation of polymers by bacteria.

The anaerobiosis without N conditions (ANA-N) produced the highest increase in CO_2 during the first 7 days for both PP and PS, and the PP ANA-N maintained a higher CO_2 production during the 28 days of the experiment, reaching a concentration of 0.25 %. These data indicate that the bacteria responsible for degradation can be active in both aerobiosis and anaerobiosis, and degradation seems to be more active in anaerobioses, which may suggest the importance of nitrogen fixers in the consortium since anaerobic conditions should favour the presence of nitrogen-fixers, resembling the anaerobic conditions present in the larvae gut. The N-fixing activity within the gut of PS-fed *T. molitor* and the negative effect of the addition of antibiotic activity on N-fixation has been described before [42]. Complementing these previous results on the relevance of N-fixation within the larvae, our results demonstrate the

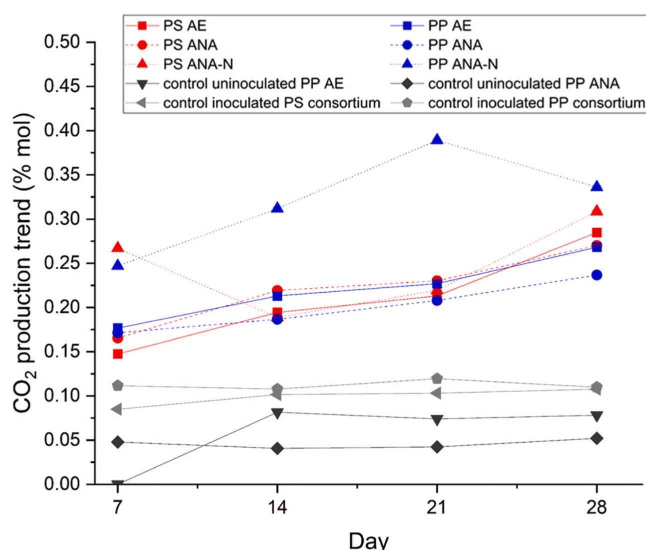

Fig. 3. Growth of different bacterial consortiums obtained through enrichment culture techniques on PS and PP as assessed by CO_2 production for 28 days. Control uninoculated PP AE/ANA (dark grey symbols): controls for aerobiosis and anaerobiosis conditions with plastic without bacteria; control inoculated PS/PP consortium (light grey symbols): controls containing PS or PP consortiums without plastic. Values are the average of 3 replicates.

relevance of N-fixers in plastic degradation in bacterial consortia outside the larvae. A decrease in CO_2 production was observed in the most active consortium, PP ANA-N, during the last week (Fig. 3), indicating that the bacterial population may have entered the stationary phase due to other nutrient limitations, such as P or Fe. On the contrary, an increase in CO_2 production rate was observed during the last week for the other conditions. This agrees with the hypothesis that the more bacteria that colonize the plastic, the more efficient and faster the degradation will be.

At the end of the respirometry test, small amounts of the PS were removed from the bottles and analyzed by SEC for the two conditions producing the highest CO_2 concentrations (PS AE and PS ANA-N). The effect of bacterial consortia on the length of polymer molecules was very similar in the two conditions, showing a limited but significant molecular weight decrease from the values of reference PS (M_n=92,400 Da, M_w=240,700 Da, and DPI=2.61) down to M_n= 87,800 Da, M_w= 223,000 Da, and DPI= 2.54, and M_n= 86,300 Da, M_w= 220,000 Da, and DPI= 2.55, for PS AE and PS ANA-N, respectively. These results confirm a process of depolymerization that is more extended than that observed in the frass of the *Z. atratus* PS-fed group, as expected for a longer enzymatic exposition. After the preferential metabolization of lower molecular weight molecules, also the higher fractions were exposed to partial depolymerization, with a decrease in both M_n and M_w. Similar behaviour was previously observed for a bacterium isolated from *T. molitor* [15]. Finally, the absence of any effect related to the incubation conditions, i.e. anaerobic vs. aerobic, on the observed degradation of PS is also coherent with the anaerobic environment found in the larvae intestine.

3.4. Changes in gut microbiome and enrichment cultures

The microbial composition of the gut of the larvae grown on bran, PS, and PP, together with the enrichment cultures maintained in aerobiosis and anaerobiosis with and without combined nitrogen, were analyzed using Illumina NGS technology. The enrichment consortia were analyzed at weeks 8 and 12 (Table S1). The number of DNA reads obtained ranged from 58×10^3 to 98×10^3. The number of OTUs was lower in the gut of bran-fed larvae (66) than in PP-fed (127) and PS-fed (99), which was also reflected in lower diversity indexes in the gut of

bran-fed larvae in comparison to plastic-fed larvae. An increase in the Shannon index of gut microbiome in plastic-fed larvae of *Z. atratus* in comparison with bran-fed larvae has already been described [25,28] and in *T. molitor* fed PP surgical masks [29] but other authors have reported an equal or even lower Shannon index when *Z. atratus* [33] or *T. molitor* [26] larvae are transferred to a plastic diet. The observed increase in the diversity indexes indicates that a high number of bacterial species may be required to degrade the synthetic polymers efficiently. This further supports the approach of using enrichment bacterial consortia instead of single species for *ex-situ* plastic degradation by the larvae microbiota.

Bran-fed larvae microbiota was dominated by *Gammaproteobacteria* of the order *Enterobacteriales*, followed by *Lactobacillales* (Fig. 4a). The presence of *Lactobacillales* increased in the gut of plastic-fed larvae to the detriment of *Enterobacteriales*, with a significant increase in the presence of *Corynebacteriales* in both PP and PS-fed larvae (Fig. 4a). The opposite trend, with an increase in *Enterobacteriales* has been reported for PP-fed *Z. atratus* [27], indicating the wide spectrum of bacteria involved in the degradation process, depending on insect larvae origin or characteristics of the polymer. Corynebacteria have already been associated with the degradation of polymers [43], more specifically with PS. Sun et al. [44] found *Corynebacterium* as the main lineage in the faeces of *Z. atratus* fed with PS, being moderately abundant in their gut [44]. In this same study, they detected the styrene monooxygenase styA in *Coryebacterium*, an enzyme described to be involved the vinyl side chain degradation [44]. When the gut microbiota was studied at a genus level (Fig. 4b), an interesting shift was observed in *Enterobacteriales,* depending on the diet. Bran-fed larvae showed a predominance (>90 %) of *Serratia*, which disappears when the larvae are transferred to plastic. The genera *Citrobacter, Hafnia* and *Enterobacter* were greatly increased in PP-fed larvae, while only *Citrobacter* and *Hafnia* increased at the expense of *Serratia* in

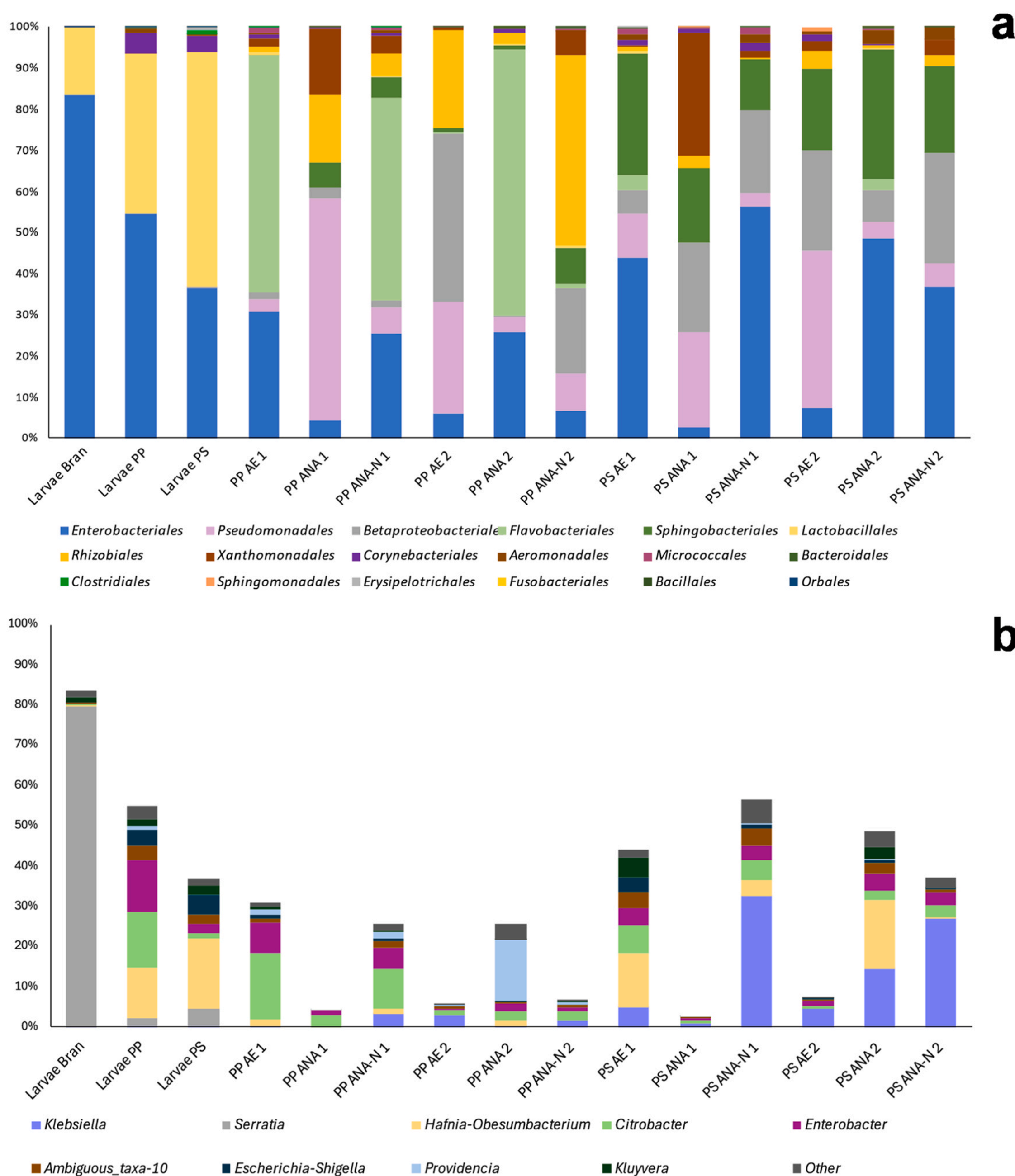

Fig. 4. : Microbial communities present in larvae gut and enrichment consortia at 8 and 12 weeks (sample 1 and 2, respectively), at order level (a) and at genus level within the *Enterobacteriales* order (b). AE: aerobic conditions; ANA: anaerobic conditions; ANA-N: anaerobic conditions without a combined nitrogen source.

PS-fed larvae.

Important differences are reported in the literature regarding the shift in gut microbiota depending on the polymer diet. Similar to our observations, a decrease in bacteria present in standard, natural polymer (bran, beeswax) diets such as *Spiroplasma* sp. and *Cronobacter* sp. for *T. molitor* [16], or *Lactobacillaceae* for *Z. atratus* [19] was found in favour of the emergence of other genus, such as *Citrobacter* and *Enterobacter* in *Z. atratus* fed PP, with *Pseudomonas, Spiroplasma* and *Corynebacterium* also associated to PP degradation [19]. Jiang et al. [13] and Yang et al. [19] described that the Firmicutes dominated the microbiota in bran-fed *Z. atratus*, their abundance decreasing in favour of representants of the genus *Ralstonia*, *Romboutsia* and, later, *Kluyvera* [13] and unclassified *Enterobacteriaceae* in PP-fed *Z. atratus*. A recent report also recorded an increase in an unclassified *Enterobacteriaceae* and *Enterococcus* in *Z. atratus* fed low molecular weight PP [27]. On the contrary, smaller changes associated with a PS diet have been reported [25] in which *Spiroplasma*, *Klebsiella*, *Lactococcus*, an unknown *Enterobacteriaceae* and *Chronobacter* dominated the microbiota of both, bran and PS-fed larvae and only minor increases in an unknown *Enterobacteriaceae*, *Enterococcus*, *Dysgonomonas* and *Sphingobacterium* were observed [14,25]. Important differences in the gut microbiota associated with bran and PP-diet have also been reported for *T. molitor* by different authors [13, 14,16,19,26,29,45]. These differences in the gut microbiome associated with bran-fed *Z. atratus* and changes associated with the plastic diet among different experiments may be due to differences in the initial microbiomes associated with different strains of the worms. The different PS degradation capabilities of *Z. atratus* strains have already been described [24], that could also be attributed to the differences in the microbial endowment of the larvae. These differences in the gut microbiome of the different larvae strains after acclimation to the plastic diet indicate that plastic degradation capability is not linked to specific species or genera but that different groups of bacteria or guilds can provide the different metabolic activities required for the degradation.

Unlike reported by other authors [33], our enrichment cultures maintained high diversity indexes (Table S1). The microbial composition of the bacterial enrichment consortia for the two polymers was more diverse than the corresponding gut microbiomes (Fig. 4a,b). The principal coordinates analysis (PCoA) revealed that the PP enrichment cultures were more distant from the initial gut microbiome than the PS enrichment cultures. However, time caused a convergence in the composition of PP and PS enrichment cultures (Fig. 5). It was previously reported that the gut microbiome of different larvae species tends to cluster with time when fed PS [13]. The naked eye could appreciate the differences in the consortium composition since the PS consortium presented a mucilaginous aspect, undoubtedly favoured by the PS fibres, and a black colour could be observed during the initial stages of enrichment both in aerobiosis and anaerobiosis. In contrast, the PP enrichment cultures were less dense and coloured, and bacteria grew in a dispersed way (Fig. S4). The PCoA also showed that the type of polymer and sampling time are more influential factors on the bacterial composition of the enrichment consortium than incubation conditions (aerobiosis/anaerobiosis and presence of combined N).

Regarding the microbial composition of enrichment cultures', *Lactobacillales*, an important part of the gut microbiomes, completely disappeared from the enrichment cultures, both in PP and PS-fed larvae (Fig. 4a). The N-fixing orders *Rhizobiales* and *Clostridiales* were present in all the enrichment cultures and plastic-eating worms but absent in the bran-eating worms, indicating that bran should contain a significant amount of combined nitrogen to sustain the bacterial metabolism. *Rhizobiales* were dominant in PP-anaerobiosis without N but not in the anaerobic cultures with NO_3, indicating a significant increase in N fixation activity due to combined N deprivation. A lower presence of *Rhizobiales* was observed in the PS enrichment cultures. This may indicate a lower N requirement due to a lower plastic degradation ability, as demonstrated by the lower CO_2 production of these enrichment cultures (Fig. 3). Previous reports have observed an increase in the abundance of members of the N-fixing genus *Klebsiella*, *Pseudomonas, Citrobacter,* and *Kluyvera* in PS-fed *T. molitor* gut, but also an important presence of N-fixation genes previously described in uncultured nitrogen-fixing bacteria [42]. These results indicate that a deeper insight in the role of

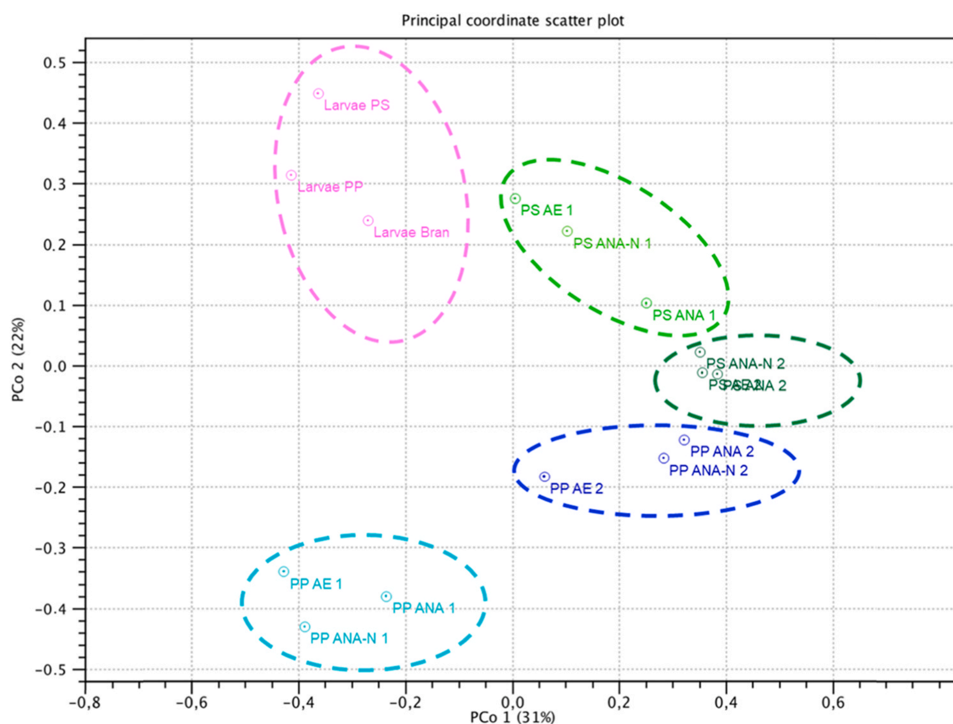

Fig. 5. Principal coordinates analysis showing the similarities between the gut microbiomes of *Z. atratus* fed on bran (Larvae-Bran), PP (Larvae-PP) or PS (Larvae-PS) and the derived enrichment cultures maintained with PP or PS as the only carbon source in aerobioses (AE), anaerobioses (ANA) and anaerobioses without combined nitrogen (ANA-N). The microbial composition of the enrichment cultures was analyzed after 8 and 12 weeks (samples 1 and 2, respectively).

N-fixing bacteria in polymer degradation is required.

The presence of *Pseudomonales*, a group widely related to plastic degradation [7,23,46] was relevant in all enrichment consortia, and, as expected, the prevalence was higher in the aerobic conditions (Fig. 4). *Flavobacteriales* seem to play an important role in PP degradation since this order is dominant in several PP-enrichment cultures. The genus *Chryseobacterium*, belonging to the *Flavobacteriales*, also dominated bacterial consortiums adapted to PP, PS and HDPE [33]. The final PS enrichment cultures were highly similar among them, with a dominance of *Enterobacteriales, Betaproteobacteriales* and *Sphingobacteriales* in anaerobiosis and a decrease in the abundance of *Enterobacteriales* to the benefit of *Pseudomonas* in aerobiosis. Enterobacteriales dominated our PS enrichment cultures. *Pseudomonales, Betaprotebacteriales* and *Sphingobacteriales* (Fig. 4a), and within the *Enterobacteriales*, the genus *Klebsiella, Hafnia* and *Citrobacter* were dominant in most conditions (Fig. 4b). A similar result was obtained for cultivable bacteria isolated from a PS enrichment culture obtained from *T. molitor* larvae capable of PS degradation that belonged to the genus *Klebsiella, Serratia, Stenotrophomonas, Citrobacter, Bacilllus, Pseudomonas, Enterococcus* and *Enterobacter* [8]. Nevertheless, the high diversity observed in the enrichment cultures, which contrasts with a much lower diversity among isolated strains, indicates the need for more specific isolation techniques for isolating plastic-degrading bacteria.

A differential abundance analysis assessed the most significant changes produced during consortium maturation by comparing the samples obtained after 8 and 12 weeks. N-fixing *Rhizobiaceae* increased with time in both plastic enrichment communities (Fig. 6). However, major changes in abundance differed depending on the type of plastic, with *Xanthobacteraceae* and *Sphingomonadacea* favoured in PS and *Pseudomonaceae* and *Burkholderiaceae* favoured in PP. In a recent work, bacterial enrichment cultures obtained from *Z. atratus* gut bacteria showed the highest increase in the genus *Alcaligenes* (*Burkholderiales*) for PS and *Chryseobacterium* (*Flavobacteriales*) for PP [33]. Again, the evolution of gut microbiota and enrichment cultures seem to be highly variable, probably dependent of larvae strain and enrichment culture conditions, reinforcing the hypothesis that multiple bacteria are involved in the degradation process.

A separate experiment was performed to assess the role of the gut microbiota in PP degradation. Gentamicin could not completely suppress gut microbiota that decreased from 5.8×10^7 UFC mL^{-1} in the control larvae to 1.8×10^7 UFC mL^{-1} in the antibiotic-treated larvae at the end of the experiment (Fig. S5a). The shift to the PP diet produced an important decrease in the concentration of cultivable bacteria in the gut. Survival (Fig. S5a), larvae weight loss and PP consumption (data not shown) were lower in the antibiotic-treated groups, but these differences were not statistically significant. On the contrary, an important shift in

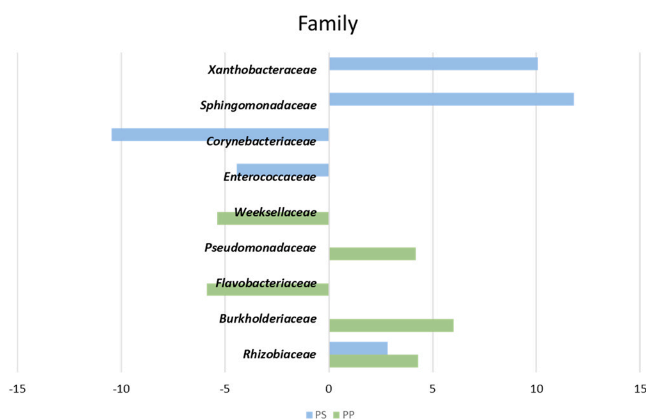

Fig. 6. Differential abundance analysis of PP and PS enrichment cultures with time. Enriched and depleted families of enrichment cultures after 12 weeks compared to 8 weeks.

gut microbiota composition was observed (Fig. S5c). As in the previous experiment, the initial gut microbiota was dominated by *Lactobacillales, Enterobacterales,* and, in this case, an important proportion of *Micrococcales* that were minoritarian in the first experiment (Fig. 4a). The transfer to a PP diet for 21 days did not affect significantly the gut microbiota composition in this experiment. The antibiotic treatment almost eliminated the *Enterobacterales* and *Micrococcales*, which was accompanied by a significant increase in *Entomoplasmatales, Flavobacteriales* and *Erysipelotrichales* in two replicates. Despite this important shift in the gut microbiota composition, only small changes in survival or PP consumption were recorded, indicating that different groups of bacteria can contribute to the larvae's degradation capacity, producing similar results. In the view of these results, the discrepancies in the role of gut bacteria in plastic degradation found in the literature may be derived from the differential effect of the antibiotic treatment, that, as in our case, may not be able to fully suppress the gut microbiota. The composition of the frass was similar to the gut composition in the control cultures, except for an important increase in the *Corynebacteriales*, an order that belongs to the *Actinomycetota*, a phylum of Gram-positive bacteria that are active degraders of organic matter in the soil and sediments and have also been described to be able to degrade artificial polymers [47]. Therefore, its increase in the frass of PP-fed larvae should be further investigated due to its biotechnological potential.

3.5. Ex-situ degradation of PS

Ex-situ degradation assays were performed using 4-week bacterial consortia. For the surface-degradation assays, bacterial consortiums were incubated for 28 days at 20 ºC in a newly designed surface contact system within a 12-wells cell-culture plates, in which the bacteria were cultivated in a small volume of saline solution over an agar layer and covered with a PS disk. Bacterial colonization was visualized by SEM and CSLM measurements (representative examples are shown in Fig. 7), whereas PS structural changes were monitored by ATR-FTIR spectroscopy.

SEM micrographs of the surfaces of PS disks showed a discontinuous coverage of bacteria. Colonies have dimensions in the micrometer length scale, but significant bacteria-free areas exist between one grouping and another (Fig. 7a). Also, CSLM (Fig. 7b) revealed an inhomogeneous colonization, possibly affected by surface roughness [48]. The analysis of the PS structural changes due to the action of bacterial consortia was carried out after an optimized cleaning process of the plastic surface, detailed in the Supplementary Material, which avoids the interference of organic contamination derived from biofilm formation. The presence of these organic residues can lead to the misattribution of degradation products, as previously reported [49]. In the ATR-FTIR spectra of incubated PS, no new peaks can be seen in the –OH region between 3500 and 3000 cm^{-1}, independently from the type of consortium inoculated. On the other hand, in the case of the PS incubated with consortia named PS AE, PS ANA and PP ANA, new absorptions, at 1715 cm^{-1} and 1734 cm^{-1}, already appeared after 14 days of incubation and were more intense at 28 days (Fig. 8). They can be associated with the development of carbonyl groups, i.e. ketones and aldehydes, respectively, as already revealed in the bio-oxidation process of PS and other plastics [7,16,23]. These results suggest a metabolic route in which an enzymatic C-C cleavage leaves these carbonyls as chain terminals in at least one of the two polymeric chain fragments resulting from enzyme dissociation. The differential mode of degradation observed in the surface-contact degradation assays in comparison to that observed in the frass may indicate that different biochemical routes are being utilized in the anaerobic gut and in aerobic conditions for the degradation of the polymer. Furthermore, the observation of variable values of the new absorptions in different surface points, with areas in which almost no spectral changes were detectable, seems to confirm the inhomogeneous colonization revealed by microscopy (Fig. 7b).

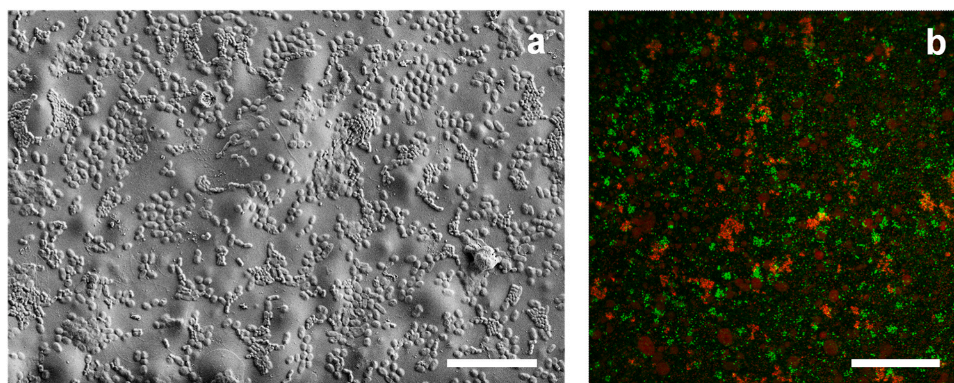

Fig. 7. SEM micrograph (a) and CLSM image after LIVE/DEAD staining (b) of PS disk surface after 28 incubation with a bacterial consortia. Scale bar: 10 µm (a) and 50 µm (b), respectively.

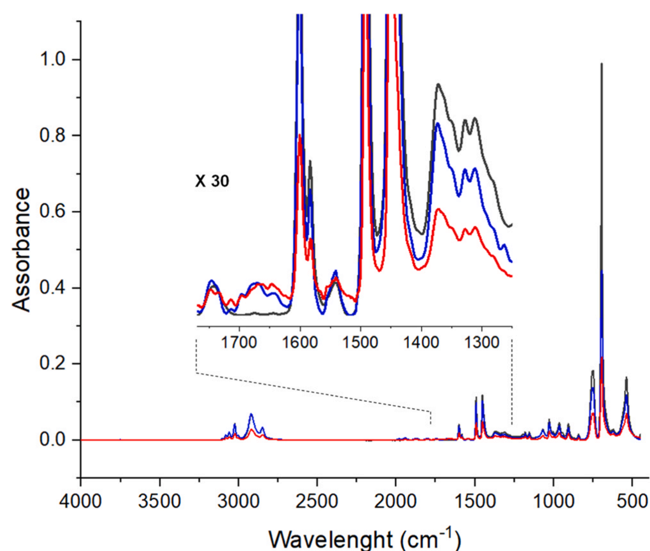

Fig. 8. ATR-FTIR spectrum of reference PS disks (grey), and PS after 14 days (blue) and 28 days (red) exposure to the enrichment cultures obtained in anaerobioses with PP as carbon source (PP ANA).

No structural or molecular changes were detected by using bulk techniques such as NMR or SEC, respectively, also when the samples were obtained by gently scraping the PS surface with a scalpel. The difficulty of detecting signs of biodegradation by these techniques indicates a degradation mechanism that is limited to just few micrometers, roughly corresponding to the depth of penetration in ATR-FTIR spectroscopy.

4. Conclusions

Larvae *of Z. atratus* presented a higher survival rate and plastic consumption capacity than those of *T. molitor* when fed with PP fabric from surgical masks. The survival results were even higher than those obtained with the bran diet. In addition, the higher consumption of PS than PP of *Z. atratus*, together with a lower larvae survival, may indicate that larvae are eating a higher amount of PS due to a lower digestibility.

With respect to the structural changes in the plastics ingested by *Z. atratus* larvae, the formation of new oxygen-containing functional groups, suggested in previous investigations, could not be confirmed neither by FTIR spectroscopy or through the more sensitive NMR measurements. On the contrary, SEC analysis detected molecular changes consisting in a PS depolymerization. Results indicate a random cleavage mechanism of C-C bonds, preferably from the lowest molecular weight chains.

Plastic-fed larvae exhibited changes in gut microbiota composition compared to those fed with natural diets and these differences were even more evident in the derived bacterial enrichment cultures. In particular, the composition of plastic-degrading enrichment cultures was more influenced by polymer type than by culture conditions and tend to converge over time. Respirometry tests performed with different enrichment cultures confirmed the active PS and PP degradation by bacteria. Both aerobic and anaerobic conditions supported plastic degradation, with anaerobic conditions favoring a more active plastic degradation, with a higher presence of nitrogen-fixing bacteria.

Differently from the behaviour observed for the ingested plastics, PS incubated with some bacterial consortia in aerobiosis showed limited signs of oxidation, as revealed by ATR-FTIR spectroscopy. This may indicate that different biochemical routes are being utilized in the anaerobic gut and in aerobic conditions for the degradation of the polymer.

In conclusion, these findings underscore the multifaceted nature of plastic degradation, implicating a diverse array of microbial species or guilds in this process. Understanding the dynamics of microbial communities and the mechanisms underlying plastic degradation is imperative for the development of effective strategies for plastic waste management.

Environmental implication

Our study investigates the fate of two polymers ubiquitously present in plastic residues as PS foams and PP fabric (the main component of the most common disposable masks used during the COVID-19 pandemic) by *Z. atratus* larvae and their gut microbiota enrichment cultures. We deepen the knowledge of the complex mechanism of polymer degradation, showing that moving from the anaerobic environment into the larvae gut to the aerobic conditions of bacterial enrichment cultures, the biodegradation route may differ. The understanding of the dynamic of microbial communities controlling polymer degradation paves the way for innovative solutions for plastic waste management.

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CRediT authorship contribution statement

Edoardo Miravalle: Writing – original draft, Visualization, Software, Investigation, Data curation. **Sabela Balboa:** Writing – review & editing, Visualization, Resources, Formal analysis. **Marco Zanetti:** Supervision, Funding acquisition. **Ana Otero:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Massimo Lazzari:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Methodology, Funding acquisition, 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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.135475.

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