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Research Article

Enhancing the biodegradability and environmental impact of microplastics utilizing *Eisenia fetida* earthworms with treated low-density polyethylene for sustainable plastic management

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Abstract
Low-density polyethylene (LDPE) is widely used in food packaging and agricultural mulching, but its disposal generates macro, meso and microplastics that infiltrate the food chain and carry harmful substances. The present study aimed to improve remediation strategies for soils contaminated with LDPE and enhance the survivability of *Eisenia fetida*. The study dissolved LDPE in trichloroethylene and treated it with starch, hydrogen peroxide, nitric acid and acetic acid, initiating thermo-oxidative reactions. The treatment decreased LDPE's crystallinity index from 48.48% to 44.06% (single treatment), 44.06% to 40.02% (double treatment) and 40.02% to 32.98% (triple treatment), achieving a 15.5% reduction in crystallinity. LDPE microplastics with 40.02% crystallinity showed lower mortality rates in *Eisenia fetida* earthworms compared to those with 44.06% and 32.98% crystallinity and untreated LDPE. When introduced to *E. fetida*, microbiota in the earthworm casts included unidentified species from *Pseudomonas* and *Zoopagomycota*, known polyethylene degraders. Microbial analysis of treated LDPE microplastics showed changes in gut microbiota, including potential degraders from *Aeromonas* and *Malassezia restricta*. XRD (X-ray diffraction techniques analyses) and FTIR(Fourier Transform Infrared Spectroscopy) analyses provided insights into distinct LDPE degradation patterns, identifying hydroxyl and carboxylic groups as functional groups. The study also investigated the ability of altered microflora with treated microplastics to degrade LDPE, favouring decreased earthworm mortality rates. The crystallinity index of treated polyethylene further reduced from 40.02% to 23.58% after 21 days of exposure to *E. fetida*. This research advances the understanding of oxidised plastics' ecological impacts and will help to develop environmentally sustainable and biodegradable LDPE.

Keywords: *Eisenia fetida,* Low-density polyethylene, Microplastics, Thermo-oxidative reaction

INTRODUCTION

Soil, as described by Ritz *et al*. (2004) as the "Biological engine of the earth", is crucial for terrestrial life. However, the Agricultural Revolution during the early 1900s brought about farming practices aimed at boosting productivity, inadvertently causing a decline in soil quality and health. The Green Revolution in India shifted towards inorganic mulches, notably polyethylene plastic mulch, to conserve water, increase yields and reduce weed growth (Kumari *et al*., 2019). Unfortunately, the widespread adoption of plastic mulch has led to the accumulation of discarded plastic fragments in fields, which break down into microplastics under certain environmental conditions. When exposed to commercial fertilizers, soil generates microplastics from plastics (Dang *et al*., 2018).

Significantly, the massive production of plastic between 1950 and 2019 resulted in 450 million tonnes of plastic, with 80% becoming waste, only 9% being recycled, 12% incinerated and 79% ending up in landfills, water bodies and oceans (Ambika *et al*., 2015, Hannah *et al*., 2023). In its solid state, LDPE molecules form dense, semi-crystalline structures. They are highly hydrophobic, meaning only the surface with limited free chain ends is accessible for enzymatic activity. This restricted surface area hinders water and potentially reactive molecule diffusion produced by microorganisms. Water

diffusion is nearly absent, and even oxygen diffusion in the crystalline regions is minimal. In line with common observations, these characteristics contribute to LDPE being widely considered as a non-biodegradable material (Koutny *et al*., 2006).

LDPE on exposure to the soil forms plastic particles with a size less than 5mm called microplastics (MPs). Microplastics known for their extensive surface area and strong hydrophobic properties can effectively absorb environmental pollutants (Horton *et al*., 2017). Consequently, soils containing microplastics may experience changes in their physicochemical characteristics, plant growth, yield and microbial activities. Microplastics and harmful substances like plasticizers and antibiotics found in plastics facilitate their movement into deeper soil layers. This is supported by the substantial specific surface area of microplastics, which promotes adsorption and contributes to escalating soil pollution.

Earthworms, a significant biomass component in soil ecosystems are active in soil structure maintenance, nutrient cycling and water regulation (Park *et al*., 2019). They also serve as bioindicators helping assess microplastics' detrimental effects at varying concentrations. These organisms prefer ingesting microplastics due to their ease of ingestion particularly favouring smaller particles ranging from 0.1 to 5 mm (Cui *et al*., 2022). According to the research conducted by Adhikari *et al*. (2023), earthworms show minimal to no adverse effects from low concentrations of LDPE. Therefore, the present study aims to enhance our understanding of the potential impacts on earthworm populations resulting from the increasing use of plastics, both presently and in the future. Specifically, it investigates earthworms exposed to elevated LDPE levels to evaluate potential effects on their mortality rates and gut and cast microbiota changes.

The introduction of polyethylene infused with starch and oxidants shows promise in mitigating plastic pollution through subsequent microbial degradation facilitated by earthworm gut and cast biota. Studies conducted by Koutny *et al*., 2006 demonstrated significant degradation of highly pre-oxidized LDPE film in soil or compost settings, with a degradation period of approximately one year, as supported by current research findings.

Eisenia fetida, an epigeic earthworm native to Europe (Zhou *et al*., 2021) can ingest treated and untreated microplastics. These earthworms were chosen for their potential to transport microplastics into the soil through horizontal burrowing activities. Interestingly, the degradation of treated polyethylene is notably enhanced within the earthworm. Hence, the rapid degradation of treated polyethylene mainly occurs in the gut of earthworms, supported by microflora. This enhancement is evident in isolated in vitro cultures of gut and cast fungal and bacterial mediums, highlighting the symbiotic connection between the microbiome and its host.

The present work aimed to explore polyethylene infused with starch and oxidants to be accessed by microflora to degrade LDPE and impact earthworm mortality rates. It examines the reduction of crystallinity in treated polyethylene after 21 days with *Eisenia fetida*. The research highlights oxidized LDPE as a unique substrate for microbial colonization and degradation, supporting *E. fetida* survival, and suggests this as a promising method to mitigate plastic pollution through microbial degradation facilitated by earthworms.

MATERIALS AND METHODS

Materials

The polyethylene packaging material was sourced from Synpack Slide N Zip Private Limited, situated in Bangalore-58, Karnataka State, India. Trichloroethylene served as the solvent, while the oxidizing agents included hydrogen peroxide, nitric acid and acetic acid, all of Analytical grade quality. Trichloroethylene and acetic acid were obtained from the brand, S D Fine, while hydrogen peroxide and nitric acid were sourced from the brand, Fisher/Qualigens and starch was acquired from the brand, Nice. Proper protective equipment was used while managing these chemicals.

Soil and earthworms collection

For the control experiment, soil samples were gathered from the surface layer (0-10cm) of the Bangalore region at coordinates 12° 58' 20.7912'' N and 77° 34' 50.3148'' E. The soil sample was subjected to drying and sieving in the laboratory to ensure particles smaller than 5mm. Mature earthworms belonging to the species *E. fetida* (Linnaeus 1758) were obtained from Laxmi Organics, Rajasthan. These earthworms were maintained at a consistent temperature of 23°C ±4°C in dark environment for two weeks before the experiment, during which they were fed with soil containing dried leaves of *Bambusa vulgaris*.

Treatment of polyethylene

The polyethylene was dissolved in trichloroethylene and subjected to a treatment process involving 30% starch and oxidizing agents like hydrogen peroxide, nitric acid, and acetic acid (Palanna *et al*., 2023). This treatment was categorized as T for a single treatment, TT for a double treatment and TTT for a triple treatment. The treated polyethylene samples (T, TT, TTT) were left to dry under sunlight for five days. Both the treated and untreated polyethylene were fragmented into pieces and sieved through a mesh with openings smaller than 5mm to obtain microplastics.

Experimental design

For every treatment (T, TT, TTT, UPE), ten replicates were carried out under a stable temperature of 23°C

±4°C, using glass containers with perforated lids to minimize water loss. The total weight of dried leaves combined with either treated (T, TT, TTT) or untreated polyethylene (UPE) was consistently maintained at 0.1g. These containers contained a mixture of dried leaves and polyethylene ranging from 20%, 40%, 60%, 80% and 100%, while the control group contained soil and dried leaves without any polyethylene. Each container was supplied with 5 ml of deionized water.

Adult earthworms chosen for the study were kept in a dark environment for 24 hours to empty their gut contents. Subsequently, they were rinsed in water and placed in the glass containers to measure their weight. Only one placed in each container to prevent any adverse effects on the earthworms. A small amount of deionized water (1 ml) was added to the lid of each container to maintain a stable humidity for the earthworms, and the setups were kept in a dark environment.

Observations were made at intervals of 12, 24, 48, 96 and 120 hours to monitor the behaviour of the earthworms. Earthworms that showed no response when their tails were stimulated with a toothpick were considered deceased. The earthworms that survived for 120 hours in the treated polyethylene setup were further monitored for an additional 16 days compared to control earthworms. After this period, the setups were weighed. The surface of the earthworms was washed with deionized water and dried with filter paper. They were then placed in a sterile petri dish for 24 hours to collect their casts. Subsequently, the earthworms were frozen for 8 hours and their gut contents were dissected. The treated polyethylene samples within the glass containers of the surviving earthworms for 21 days were analysed using Fourier Transform Infrared Spectroscopy (FT-IR) and X-ray diffraction (XRD) techniques. Chemical changes in the structure of untreated and treated polyethylene with starch and oxidizing agents were measured using an FT-IR spectrophotometer. They were observed with a spectrum range from 4000 to 500 cm^{-1}. The change in peaks to identify the functional groups before being introduced to earthworm, FT-IR spectrophotometry was utilized to evaluate chemical modifications in both untreated and treated polyethylene treated with starch and oxidizing agents.

XRD analysis of LDPE samples was carried out using Origin 2020 software to study their patterns. The percentage of crystallinity in these samples was determined using the integration method, which involves comparing the straight background line with the area under the entire curve.

The percentage crystallinity was calculated using the formula (https://mcl.mse.utah.edu/xrd-crystallinity-by-integration/):

Percentage crystallinity=(area under the crystallinity peaks / area under all peaks)*100 Eq.1

The calculation of the Carbonyl Index (CI) aimed to gauge the extent of biodegradation. It involved comparing the relative intensities of the carbonyl group at 1712 cm^{-1} and the CH2 group at 1462 cm^{-1} (Hadad *et al*., 2005). The equation used for calculating CI is as follows:

Carbonyl Index (CI) = Absorption at 1712 cm^{-1} / Absorption at 1462 cm^{-1} Eq. 2

Following the OECD Guidelines for testing of Chemicals (OECD, 1984), after 21 days of exposure to treated polyethylene and under control conditions, the casts and guts of the earthworms were further examined for bacterial and fungal microflora by 16S RNA and ITS analysis.

Preparation of microbial medium

After exposing the earthworms to TT LDPE for 21 days, the casts and guts of the earthworms were collected. This process involved placing the earthworm on a sterile petri dish for 24 hours to collect its cast and then freezing it for 8 hours to extract its gut microflora (Dongxing *et al*., 2016). Subsequently, malt extract medium and Modified M9 medium with yeast extract were prepared to inoculate fungal and bacterial cultures, respectively, obtained from the gut and cast of the earthworm. The inoculated medium underwent a 21day incubation period with 0.1g of TT as the sole carbon source to observe its degradation in the presence of the microbiota. The treated polyethylene exposed to the fungal and bacterial was evaluated by FT-IR and XRD analysis.

RESULTS AND DISCUSSION

Mortality rate of *Eisenia fetida*

Exposing *Eisenia fetida* earthworms to untreated plastics can lead to mortality and changes in their gut microflora (Huerta *et al*., 2018). In the present study, a mortality assessment was conducted to evaluate the impact of polyethylene on these earthworms. Earthworms exhibited avoidance and selective behaviours in areas with high concentrations of plastics such a UPE, T and TTT. Observations suggest that these earthworms ingest particles smaller than 5 mm microplastics, resulting in visible physical changes and an increase in mortality. However, TT showed lower mortality rates.

The mortality rate (Table 1) was evaluated following OECD (1984) guidelines for 120 hours to ascertain LC_{50} values. The results showed that earthworms consuming polyethylene treated twice (TT) had lower mortality rates compared to those treated once (T), thrice (TTT) and untreated polyethylene (UPE). An ANOVA single-factor analysis revealed a significant difference in mortality between T, TTT, UPE and TT, with a P-

Table 1. Mortality rate of *Eisenia fetida* with LC_{50} Value on exposure to T(treated once), TT(treated twice), TTT(treated thrice) and UPE(untreated polyethylene)

LR	T	TT	TTT	UPE
Time (Hours)	**LC50 VALUE mg**	**LC50 VALUE mg**	**LC50 VALUE mg**	**LC50 VALUE mg**
24	213	213	87.1	174
48	145	213	44.7	51.3
72	135	209	28.8	24.5
96	2.75	204	27.5	12.9
120	1.25	204	26.9	12.6

value of 0.001458 indicating statistical significance. This statistical analysis confirms that the differences in mortality rates are not due to random variation but are a result of the treatments applied to the polyethylene.

XRD analysis of both treated and untreated

The change in treated polyethylene's crystallinity was examined using the XRD-pattern analysis software Origin 2020 (Fig. 1), comparing the graphs of all treated (T, TT, TTT) and untreated PE. According to Equation 1, untreated polyethylene showed a crystallinity percentage of 48.48%. Specifically, T exhibited a crystallinity of 44.06%, TT had 40.02% and TTT showed 32.98% of crystallinity. Remarkably, the earthworm *E. fetida* demonstrated a decrease in mortality when exposed to TT polyethylene for 21 days. This observation implies that the TT treated polyethylene, with its lower crystallinity and potentially higher biodegradability, is less harmful to the earthworms. The improved survival rates of *E. fetida* can be attributed to the easier breakdown of the TT treated polyethylene by the altered microflora, facilitating a safer and more manageable environment for the earthworms. Thus, the study underscores the importance of reducing crystallinity in polyethylene to enhance its biodegradability and mitigate its adverse effects on soil biota, providing a promising approach to managing plastic pollution.

The XRD analysis (Fig. 2) indicated critical insights into the crystallinity index of treated polyethylene after various exposures, highlighting the role of earthworms and their associated microflora in the degradation process. The crystallinity index of treated polyethylene decreased to 23.58% after 21 days of exposure to *E. fetida*. This initial reduction indicates that the earthworms contribute to the polymer structure's breakdown. Further exposure to fungal and bacterial culture media derived from *E. fetida* castings and gut showed even more significant reductions in crystallinity. In the fungal culture medium from castings, the crystallinity index was 21.59%, while in the bacterial culture medium from castings, it was 21.02%. Similarly, exposure to fungal and bacterial culture media from the gut resulted in crystallinity indices of 22.73% and 20.45%, respectively. These reductions suggest that both fungal and bacterial microflora play substantial roles in degrading polyethylene.

Potassium Bromide - Fourier Transform Infrared Spectroscopy (KBr-FTIR) analysis of treated polyethylene FT-IR analysis, as illustrated in Fig. 3 and detailed in Table 2, focused on changes in peaks associated with functional groups before exposure to the earthworm *E. fetida*. Specifically, the TT sample displayed functional groups like carboxylic acid, carbon dioxide and anhydride groups. This analysis is essential for understand-

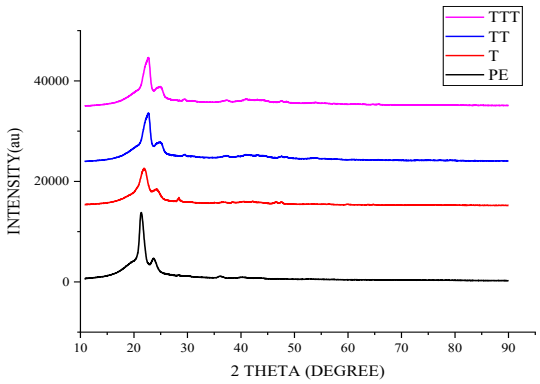

Fig. 1. *X-ray diffraction techniques analyses (XRD) analysis of polyethylene: Polyethylene dissolved in trichloroethylene treated with starch and oxidizing agents treated thrice (TTT), twice (TT) and once (T), untreated polyethylene (PE)*

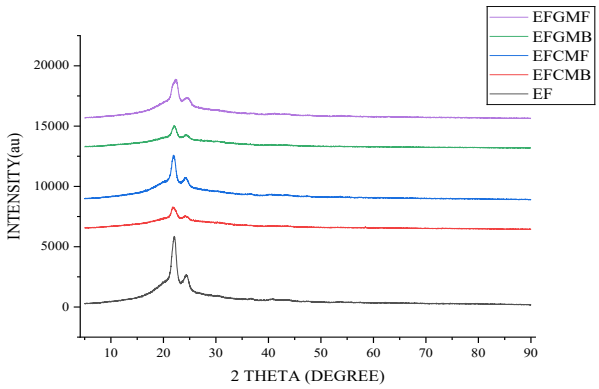

Fig. 2. *X-ray diffraction techniques analyses (XRD) analysis of polyethylene: Treated Polyethylene on exposure to Eisenia fetida gut fungi (EFGMF), Eisenia fetida gut bacteria (EFGMB), Eisenia fetida cast fungi (EFCMF), Eisenia fetida cast bacteria (EFCMB), Eisenia fetida (EF)*

ing the chemical modifications induced by the treatment process. Notably, the TT (double-treated) sample displayed functional groups such as carboxylic acids, carbon dioxide and anhydrides.

Due to the treatment process, these functional groups indicate significant oxidative modifications in the polyethylene structure. Carboxylic acid groups are known to enhance the hydrophilicity of polymers, making them more accessible to microbial attack and subsequent biodegradation (Hakkarainen *et al*., 2004). Similarly, carbon dioxide and anhydride groups suggest the occurrence of oxidative degradation, which breaks down the polymer chains and creates reactive sites for microbial colonization (Ojeda *et al*., 2009).

These chemical modifications are crucial for biodegradation, increasing the polyethylene's susceptibility to microbial action. The introduction of these functional groups contributes to the observed reduction in the crystallinity index of the treated polyethylene, as confirmed by XRD analysis. Reduced crystallinity enhances the polymer's degradability, supporting the survival and activity of *E. fetida* (Sivan, 2011).

Potassium Bromide - Fourier Transform Infrared Spectroscopy (KBr- FTIR) analysis provides critical insights into the chemical changes induced by the treatment process. The identification of functional groups such as carboxylic acids, carbon dioxide, and anhydrides in the TT sample underscores the effectiveness of the treatment in promoting polyethylene biodegradability. These chemical transformations are pivotal for reducing the environmental impact of polyethylene and enhancing its degradation by soil organisms like *E. fetida*.

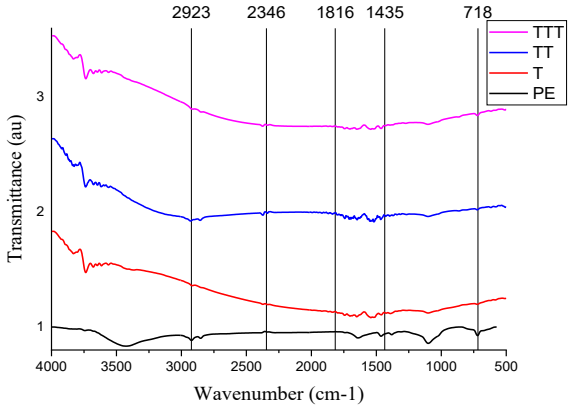

Fig. 3. *Potassium Bromide - Fourier Transform Infrared Spectroscopy KBr-FTIR(KBr-FTIR) analysis of polyethylene; Polyethylene dissolved in trichloroethylene treated with starch and oxidizing agents treated once(T), twice(TT) and thrice(TTT), untreated polyethylene (PE)*

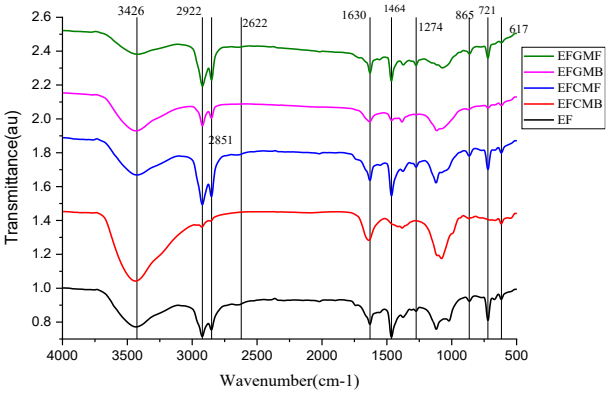

Fig. 4. *Potassium bromide - Fourier Transform Infrared Spectroscopy (KBr-FTIR) analysis of polyethylene; Polyethylene exposed to the Eisenia fetida gut Fungi (EFGMF); Polyethylene exposed to Eisenia fetida gut Bacteria (EFGMB). Polyethylene exposed to Eisenia fetida cast fungi (EFCMF); Polyethylene exposed to Eisenia fetida cast Bacteria (EFCMB); Treated polyethylene subjected to Eisenia fetida (EF)*

Potassium Bromide - Fourier Transform Infrared Spectroscopy (KBr- FT-IR) analysis of treated polyethylene after exposure to *Eisenia fetida*

As illustrated in Fig. 4 and detailed in Table 3, FT-IR analysis was performed on treated polyethylene (TT) to detect shifts in peaks indicative of changes in functional groups following exposure to *E. fetida* earthworms for 21 days. Polyethylene treated twice (TT) was introduced into bacterial and fungal environments containing gut or cast microbial cultures, followed by a 21-day incubation period. These conditions revealed alterations in functional groups such as alcohol, carboxylic acid and amine groups.

These functional groups indicate significant biochemical interactions between the treated polyethylene and the microbial environment. Alcohol and carboxylic acid groups suggest oxidative degradation, which increases

Table 2. FTIR peak position, functional group,class and peak details in treated polyethylene(T,TT,TTT) and untreated polyethylene(PE)(https:// instanano.com/all/characterization/ftir/ftir-functional-group-search/)

Peak Position(cm^{-1})	Functional group	Class	Peak Details
718	C-Cl stretching	halo compound	strong
1435	O-H bending	carboxylic acid	medium
1816	C=O stretching	Anhydride	strong
2346	O=C=O stretching	carbon dioxide	strong
2923	O-H stretching	carboxylic acid	strong, broad

Table 3. FTIR peak position, functional group, class and peak details in treated polyethylene subjected to microbial medium (https:// instanano.com/all/characterization/ftir/ftir-functional-group-search/)

Peak Position(cm^{-1})	Functional Group	Class	Peak Details
3426	O-H stretching	Alcohol	strong, broad
2922	O-H stretching	carboxylic acid	strong, broad
2922	N-H stretching	amine salt	strong, broad
2851	O-H stretching	carboxylic acid	strong, broad
2851	O-H stretching	Alcohol	weak, broad
2851	N-H stretching	amine salt	strong, broad
2622	O-H stretching	carboxylic acid	strong, broad
1630	C=O stretching	secondary amide	strong
1630	C=O stretching	tertiary amide	strong
1630	C=O stretching	δ-lactam	strong
1464	C-H bending	Alkane	medium
1464	C-H bending	Alkane	medium
1274	C-N stretching	aromatic amine	strong
1274	C-O stretching	aromatic ester	strong
865	C-H bending	1,2,4-trisubstituted	strong
865	C-H bending	1,3-disubstituted	strong
721	C-Cl stretching	halo compound	strong
721	C=C bending	Alkene	strong
617	C-Cl stretching	halo compound	strong

the hydrophilicity of the polymer, thereby enhancing its susceptibility to microbial attack (Sivan, 2011). On the other hand, amine groups indicate potential interactions with nitrogenous compounds in the microbial environment, further promoting biodegradation (Ojeda *et al*., 2009).

The modification of carbonyl groups upon exposure to a microbial environment serves as active sites for the photolytic cleavage of the polymer backbone. This photodegradation mechanism is crucial, as it initiates the breakdown of long polymer chains into smaller, more biodegradable fragments (Koutny *et al*., 2006). The existence of these functional groups facilitates conformational changes on the polymer surface, making it more accessible to microbial colonization and subsequent degradation.

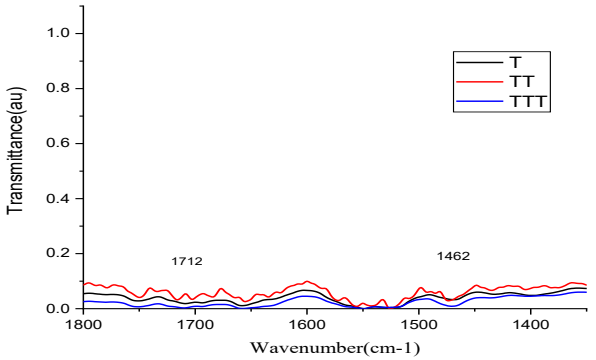

Fig. 5. *Potassium Bromide - Fourier Transform Infrared Spectroscopy (KBr-FTIR) analysis of polyethylene for Carbonyl Index. Polyethylene dissolved in trichloroethylene treated with starch and oxidizing agents treated once(T), twice(TT) and thrice(TTT)*

This study suggests that the treated TT polyethylene is particularly susceptible to degradation by microorganisms, emphasizing that PE oxidized in this manner is prone to degradation. These findings align with previous research indicating that oxidative treatments enhance the biodegradability of polyethylene by creating reactive sites for microbial attack (Koutny *et al*., 2006). FT-IR analysis demonstrates significant shifts in functional groups following microbial exposure, highlighting the increased degradability of treated TT polyethylene. The importance of these functional groups in facilitating the biodegradation process, supports the hypothesis that oxidized PE is more environmentally sustainable.

FT-IR Analysis for Carbonyl Index in T, TT and TTT Samples

FT-IR analysis, as illustrated in Fig. 5,6 and detailed in Table 4, was performed to track changes in the carbonyl peaks or double bonds present in the LDPE structure, signalling potential biodegradation. Exposure of

Table 4. Crystallinity Index and Carbonyl Index of LDPE microplastics

TREATMENTS	CRYSTALLINITY INDEX (%)	CARBONYL INDEX
EF	23.58	1.25
EFCMB	21.02	1.03
EFCMF	21.59	1.31
EFGMB	20.45	1.07
EFGMF	22.73	1.19

LDPE to oxidation results in the formation of carbonyl peaks, which serve as sites for bacterial attachment (Albertsson *et al*., 1990). This process is crucial because the presence of carbonyl groups enhances the susceptibility of polyethylene to microbial degradation.

FT-IR Analysis for Carbonyl Index in TT after exposure to gut and cast microflora of *Eisenia fetida*

In this study, polyethylene treated twice (TT) with starch and oxidizing agent exhibited a notable change in carbonyl index compared to samples treated once (T) or three times (TTT). This indicates increased susceptibility of TT, especially TT exposed to *E. fetida* (EF), to degradation by microorganisms. The comparative analysis of the Carbonyl Index of treated polyethylene (TT) with other samples exposed to *E. fetida* (EF), *E. fetida* cast fungi (EFCMF), *E. fetida* gut fungi (EFGMF), *E. fetida* gut bacteria (EFGMB) and *E. fetida* cast bacteria (EFCMB) for 21 days showed significant changes. This change, observed after biodegradation, indicated bacterial oxidation of carbonyl residues due to enzymatic hydrolysis.

The FT-IR results align with other studies where carbonyl peaks' formation and subsequent change elucidate the biodegradation mechanism (Montazer *et al*., 2018; Selke *et al*., 2015). For instance, Koutny *et al*. (2006) reported that LDPE oxidized in this manner is prone to degradation. In this study, the consumption of carbonyl residues by bacteria was measured in terms of the Carbonyl Index (CI), which showed a notable change after microbial treatment by bacteria and fungi (Table 4). Other studies have also observed similar results where microbial degradation was evident by changes in the CI index. (Sudhakar *et al*., 2008; Esmaeili *et al*., 2013; Maroof *et al*., 2020; Nag *et al*., 2021).

FT-IR analysis provided crucial insights into the structural changes in treated polyethylene, highlighting the role of carbonyl groups in promoting microbial degradation. The observed decrease in the Carbonyl Index after exposure to various microbial environments underscores the effectiveness of the oxidation treatment in enhancing the biodegradability of LDPE.

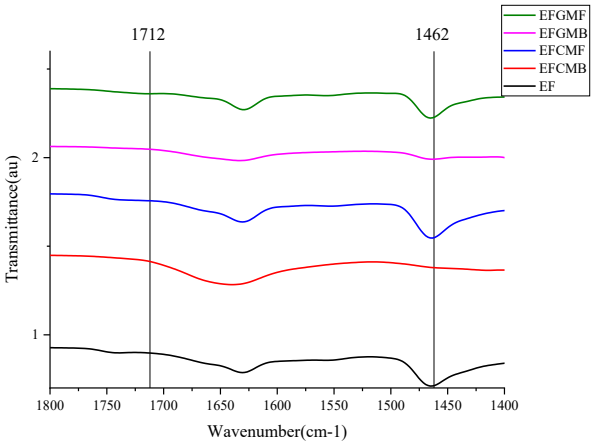

Fig. 6. *KBr-FTIR analysis of polyethylene for Carbonyl Index. Polyethylene exposed to the gut medium of Eisenia fetida fungi (EFGMF); Polyethylene exposed to the gut medium of Eisenia fetida bacteria (EFGMB); Polyethylene exposed to the cast medium of Eisenia fetida fungi (EFCMF); Polyethylene exposed to the cast medium of Eisenia fetida bacteria (EFCMB). Polyethylene subjected to Eisenia fetida (EF)*

Furthermore, the peaks observed in treated LDPE with *E. fetida* resembled those found in treated LDPE within a microbial medium, indicating the involvement of gut and cast microflora in LDPE degradation. This process is crucial for transforming LDPE into nutrients that aid in reducing the mortality rate of *E. fetida*. The microflora metabolises or interacts with treated TT polyethylene in ways that generate similar chemical compounds or functional groups, suggesting a potential role of gut microflora in TT polyethylene's degradation or modification. The capacity of gut microflora to metabolize treated LDPE and produce products akin to those produced by earthworms could improve nutrient cycling and availability in the soil ecosystem. This, in turn, could indirectly impact earthworm survival and health by influencing soil quality and nutrient availability.

FT-IR analysis on gut microflora treated with LDPE can provide valuable insights into how microorganisms support earthworm survival on LDPE-enriched diets. This analysis offers initial evidence of microbial metabolism and chemical alterations that may impact soil health and ecosystem dynamics. It highlights TT's vulnerability to degradation by microorganisms when exposed to *E. fetida* earthworm and its gut and cast microbiota, suggesting that LDPE oxidized in this manner is prone to decomposition. The microbiota plays a role in decomposition, offering nutritional support for earthworm survival.

Microbial analysis

Structure of gut bacterial communities

Table 5 presents the bacterial communities in the gut of *E. fetida* earthworms. They were examined through

Table 5. Composition of bacterial phyla in the gut and cast of the earthworm *Eisenia fetida* exposed to TT

	TREATED GUT	CONTROL GUT	TREATED CAST	CONTROL CAST
Phylum	**%**	**%**	**%**	**%**
Proteobacteria	99.27474	93.27113	82.38502	75.6902
Firmicutes	0.113468	3.145864	1.416885	4.40946
Actinobacteria	0.167277	2.343104	1.731039	3.976451
Bacteroidetes	0.414098	0.194729	12.48326	11.13768
Planctomycetes	0.022226	0.287722	0.503923	1.345242

16S amplicon sequencing. Six samples were collected, including control and polyethylene-fed (TT) groups. The sequencing yielded a total of 507,046 reads. Using QIIME analysis, we identified 1,654 Operational Taxonomic Units (OTUs) across 8 phyla, 17 classes, 38 orders, 58 families, and 81 genera. As a result, our discussion primarily focuses on presenting the outcomes obtained from QIIME.

Categorisation of bacteria by phylum to identify dominant gut communities in earthworms

The majority consisted of *Proteobacteria* (99.27%), followed by *Bacteroidetes* (0.41%), *Actinobacteria* (0.16%) *and Firmicutes* (0.11%). Notably, only *Proteobacteria*, *Bacteroidetes, Actinobacteria* and *Firmicutes* were consistently found in all samples treated with polyethylene (TT), known for their ability to degrade polyethylene (Kumar *et al*., 2021). Transitioning to the genus level, we identified a total of 397 genera, with *Aeromonas* (89.81%) and *Pseudomonas* (8.88%) being the most prevalent. Interestingly, these two genera were specifically associated with the treatment involving plastic ingestion (Park *et al*., 2019). Specifically, the percentage of *Proteobacteria* and *Bacteriodetes* increased, while the percentages of *Firmicutes, Chloroflexi, Actinobacteria* and *Planctomycetes* decreased. It is worth noting that *Proteobacteria* and *Bacteriodetes* are recognized as plastic-associated bacteria because of their role in breaking down plastic mulch in agricultural environments(Atanasova *et al*., 2021).

Structure of gut fungal communities

Table 6 presents the fungal communities in the gut of *Eisenia fetida* earthworms were studied using ITS amplicon analysis. Six samples, including control and polyethylene-fed (TT) groups, were collected, resulting in 233,571 reads from the sequencing process. Through QIIME analysis, we identified 152 Operational Taxonomic Units (OTUs) across 3 phyla, 3 classes, 3 orders, 3 families, and 3 genera. This led us to focus present subsequent discussion on presenting the outcomes obtained from QIIME.

Composition of fungal phyla in the gut of the earthworm *Eisenia fetida* exposed to TT

To improve present understanding of the primary fungal communities in the earthworms' digestive tract, the study categorized fungi by phylum. *Zoopagomycota* accounted for the largest proportion (27.10%), followed by *Basidiomycota* (24.10%). These phyla, *Zoopagomycota* and *Basidiomycota*, are well-known for their involvement in organic decomposition (Curlevski *et al*., 2010). Importantly, only *Zoopagomycota* (Lacerda *et al*., 2020) and *Basidiomycota* (Ekanayaka AH *et al*., 2022) were consistently found in all samples treated with polyethylene (TT), showcasing their ability to degrade polyethylene (Lacerda *et al*., 2020; Ekanayaka *et al*., 2022). Moving to the genus level, we identified a single genus, with *Malassezia* (24.90%) of the phylum *Basidiomycota* being the most abundant. FT-IR analysis revealed the presence of aromatic ester functional groups upon exposure of treated polyethylene to the fungal community. *Malassezia*, which is also implicated in the esterification of medium-chain fatty acids to produce antimycotic ethyl ester derivatives (Mayser, 2015), further highlights the fungal community's capability to consume TT.

Exposure of *E. fetida* earthworms to treated polyethylene (TT) led to an elevated percentage of *Zoopagomycota* and *Basidiomycota* in both their gut and cast

Table 6. Composition of microbial phyla in the gut and cast of the earthworm *Eisenia fetida* exposed to TT

	TREATED GUT	CONTROL GUT	TREATED CAST	CONTROL CAST
Phylum	**%**	**%**	**%**	**%**
Unidentified	47.9853	1.9138	0.0879	0.2319
Zoopagomycota	27.1062	35.0065	51.1855	15.0893
Basidiomycota	24.9084	0.2396	0.9572	0.0399

fungal communities. The lack of other phyla suggests that *Zoopagomycota* and *Basidiomycota* have the ability to thrive and break down treated polyethylene (TT).

Differential abundance analysis

An ANOVA single-factor analysis conducted on the gut microflora of earthworms exposed to TTPE and those not exposed yielded a p-value of 0.04517, signifying statistical significance. This implies that the gut microflora likely has a noteworthy impact on earthworm survival with PE-enriched feed, as evidenced by observed differences in survival rates or other relevant metrics between the groups.

Microbial community diversity is influenced by feeding regime

We conducted a more detailed analysis of the differences in gut bacterial communities at the Operational Taxonomic Units (OTUs) and genus levels to provide a more focused assessment of their relative composition. The rarefaction curves of the samples reached saturation, indicating that the bacterial diversity was adequately represented by the number of reads in the samples. Both the Simpson and Shannon Entropy diversity indices exhibited a similar trend across the treatments and time points. These indices are measures used to assess species diversity within a community. The value of 0.05 for samples treated with PE implies that certain species may dominate the community more than others, while the value of 0.33 for untreated PE samples suggests that species in the community are distributed more evenly, with fewer dominant species and a greater variety of species present.

The present study analysed the prevalent microbial communities in earthworm casts treated with polyethylene and observed that *Proteobacteria, Bacteroidota, Firmicutes, Verrucomicrobiota, Planctomycetes* and *Chloroflexi* were the dominant microbial taxa. Conversely, the microbial community in untreated earthworm casts was characterized by *Proteobacteria, Bacteroidota, Firmicutes, Actinobacteria, Verrucomicrobiota, Planctomycetes, Chloroflexi* and *Patescibacteria.*

Likewise, in the earthworm gut treated with polyethylene, the dominant microbial taxa included *Proteobacteria, Bacteroidota, Actinobacteria, Firmicutes, Planctomycetes, Tenericutes, Chloroflexi, Euryarchaeota.* In contrast, the untreated earthworm gut had a microbial community characterized by *Proteobacteria, Firmicutes, Actinobacteria, Chloroflexi, Planctomycetes, Bacteroidetes, Verrucomicrobia, Euryarchaeota, Fibrobacteres, Acidobacteria, Fibrobacteres, Patescibacteria, Chlamydiae, Lentissphaerea, Epsilonbacteraeota, Entotheonellaeota, BRC1, Armatimonadetes, Nitrospirae.*

In the earthworm cast treated with polyethylene, the predominant fungal taxa included *Zoopagomycota, Ascomycota, Rozellomycota* and *Basidiomycota*. Conversely, the fungal community in the untreated earthworm gut comprised *Ascomycota, Zoopagomycota, Rozellomycota, unidentified taxa, Mortierellomycota and Basidiomycota.*

In the earthworm gut treated with polyethylene, the dominant fungal taxa were *unidentified taxa, Zoopagomycota and Basidiomycota.* In contrast, the untreated earthworm gut had a fungal community characterized by *Ascomycota, Zoopagomycota, Rozellomycota, unidentified taxa, Mortierellomycota, Basidiomycota* and *Mucoromycota*. This alteration in the microbiota contributes to the adaptation of earthworms to ingest and degrade treated polyethylene (TT), thereby supporting their survival.

In the gut of *E. fetida* exposed to treated polyethylene (TT), 89.81% of the microbial population consisted of *Aeromonas* species from the *Proteobacteria* phylum and 24.90% *Malassezia restricta*. Conversely, in the cast of *E. fetida* exposed to treated LDPE, 36.95% of the microbial population comprised *Pseudomonas* species from the *Proteobacteria* phylum. These microbial populations are known for their involvement in plastic degradation, contributing to a reduction in the mortality rate of earthworms.

In Tables 5 and 6, the alterations in the microbiota of earthworms contribute significantly to their ability to ingest and degrade treated polyethylene (TT), thus enhancing their survival. The gut microflora plays a crucial role in this process, as indicated by variations in survival rates and other relevant metrics observed between the groups.

Adaptation to treated polyethylene

Earthworms *E. fetida*, with an altered microbiota are better adapted to ingest and degrade treated polyethylene. The presence of specific microbial populations in the gut that are capable of degrading plastic supports this adaptation. These microbes break down the polyethylene, making it easier for earthworms to process and thus reducing the harmful effects of ingesting plastic.

Microbial Impact on survival rates

The survival rates of earthworms *Eisenia fetida* fed with PE-enriched feed show notable differences, highlighting the importance of gut microflora. The reduction in mortality rates among earthworms consuming treated polyethylene can be attributed to the presence of plastic-degrading microbes in their gut. These microbes help break down polyethylene into less harmful substances, which aids in the overall health and survival of earthworms.

Diversity Indices Analysis

The Simpson and Shannon Entropy diversity indices provide insights into the microbial community structure. For samples treated with PE, both diversity indices

have a value of 0.05. This lower value indicates that the microbial community is less diverse, with certain species dominating. These dominant species are likely efficient in degrading polyethylene, leading to a community structure that supports the earthworms' adaptation to the treated polyethylene. For untreated PE samples, the diversity indices have a value of 0.33. This higher value suggests a more evenly distributed microbial community with a greater variety of species. No particular species dominated the community, leading to a broader range of microbial functions, though not necessarily specialized for polyethylene degradation.

These observations imply that while treated polyethylene fosters a less diverse but more specialized microbial community adept at plastic degradation, untreated polyethylene supports a more diverse microbial community. The presence of specialized plastic-degrading microbes in treated PE samples contributes directly to reducing the mortality rate of earthworms by helping them better manage and break down the ingested polyethylene. This supports the hypothesis that microbial populations play a crucial role in the survival and adaptation of earthworms to plastic-containing environments.

Conclusion

Our study challenges the long-held belief that LDPE is non-biodegradable by demonstrating that treated polyethylene exposed to the gut and cast microbial community of *E. fetida* undergoes significant degradation. Exposure to 60% treated polyethylene (TT) reduces earthworm mortality rate compared to untreated and treated polyethylene (T, TTT). XRD analysis shows a 15.5% reduction in crystallinity due to the thermo-oxidation reaction, with a further reduction of 16.44% facilitated by the gut microflora. FTIR analysis identifies hydroxyl and carboxylic groups as functional groups, with increased favourable functional groups facilitated by the gut and cast microflora of *E. fetida*. Microbial diversity analysis reveals that the *Proteobacteria* and *Zoopagomycota* phyla aid in the degradation of treated polyethylene. The genus *M. restricta* in the gut acts as a potential degrader, and a higher percentage of *Aeromona*s in treated polyethylene encourages the degradation of treated LDPE. These findings highlight the earthworm's ability to consume and decompose treated polyethylene, offering promising prospects for improving remediation strategies in soils contaminated with LDPE and enhancing the survivability of *E fetida*.

Data accessibility

The raw microbial sequence data are available from the NCBI short sequence read archive (SRA) under accession number PRJNA1105549, https://dataview.ncbi.nlm.nih.gov/object/PRJNA1105549?reviewer=usd852462pvupipukh671dgf1v.

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Conflict of interest

The authors declare that they have no conflict of interest.

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