Plastic biodegradation: do *Galleria mellonella* larvae bio-assimilate polyethylene? A spectral histology approach using isotopic labelling and infrared microspectroscopy.

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SUPPORTING INFORMATION

19 pages, 3 tables, 6 figures.

Supplementary Table 1. Average food consumptions at L3 stage.

	Food ingested for 16 days (mg per larva) (standard deviation)			Development at day 16 (except*) from L2-L3 (standard deviation)	
Diet	**Pollen**	**Beeswax**	**LDPE**	**Weight gain/loss (mg)**	**Note**
Control	0	0	0	-4.5* (0.98)	All dead by day 3
Pollen	135.4 (11.84)	0	0	+ 42.4 (11.19)	
Beeswax	0	61.62 (28.17)	0	+ 5.9 (15.4)	
Beeswax + Pollen	187.75 (21.19)	312.93 (28.92)	0	+ 181.75 (38.67)	Strongest evolution
LDPE	0	0	0.6 (0.3)	- 13.24* (8.26)	Death between day 3 and day 6
LDPE + pollen	158.83 (58.50)	0	2.53 (2.17)	+ 43.18 (30.07)	

The food consumption is given as the average consumed weight per individual larva.

Supplementary Table 2. Average food consumptions at L6 stage.

	Food ingestion for 7 days (mg per larva) (standard deviation)			Development at J7 from L6 (standard deviation)	
Diet	**Pollen**	**Beeswax**	**LDPE**	**Weight gain/loss mg**	**Note**
Nothing	0	0	0	- 48.25 (13.15)	Weight loss
Pollen	255.67 (21.99)	0	0	+ 0.33 (7.63)	No weight change
Beeswax	0	211 (67)	0	-5 (25.68)	No weight change
Beeswax + Pollen	251 (32.83)	202 (63)	0	+ 113.25 (79.32)	Optimal diet
LDPE	0	0	2.61 (1.69)	- 48.33 (16.43)	Weight loss
LDPE + Pollen	179 (47.27)	0	3.34 (1.24)	-6.5 (19.64)	No weight change

The food consumption is given as the average consumed weight per individual larva.

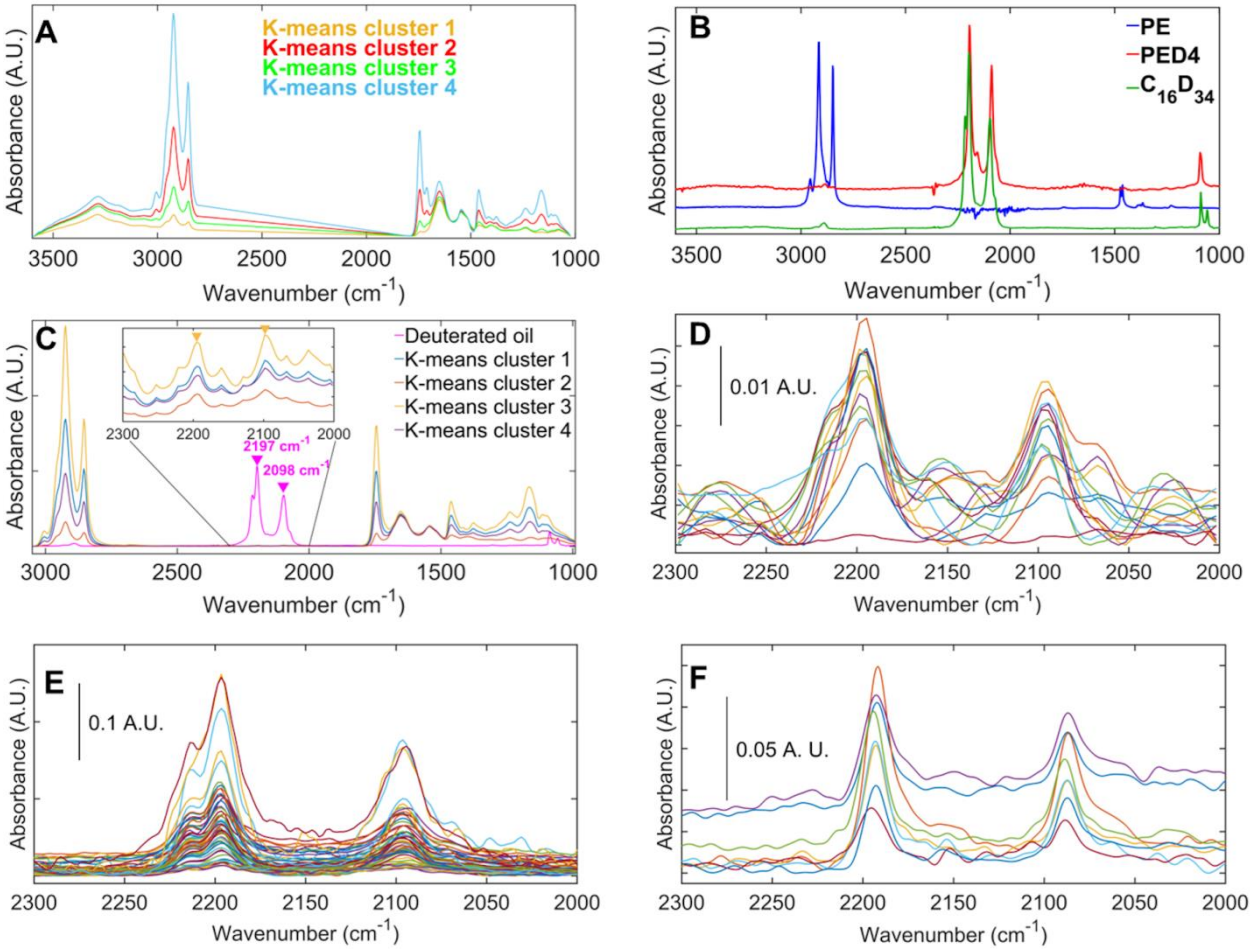

Figure S1. Infrared spectra from larval tissues and chemicals used in this study. A) Representative average spectra of different tissues from a control larva grouped in 4 clusters by k-means clustering. B) Infrared spectra of normal PE $(CH_2)_n$ in blue, of $C_{16}D_{34}$ perdeuterated oil in green, and of perdeuterated PE $(CD_2)_n$ in red showing the C-H peaks at circa 2920 and 2850 cm^{-1} in PE, and the C-D peaks at circa 2200 and 2010 cm^{-1} in the deuterated PE and the oil. Spectra were normalized and offset for clarity. C) Representative spectra from a larva fed with $C_{16}D_{34}$ oil and pollen, and $C_{16}D_{34}$ oil spectrum. Tissue spectra were grouped in 4 clusters by k-means clustering. Insert: x70 zoom on the 2300-2000 cm^{-1} range containing the C-D peaks. The fatter tissues contain more C-D. D) IR spectra extracted from the C-D rich region along the line shown in Figure 2K. Only the 2250-2050 cm^{-1} C-D peak region of the IR spectra is shown. E) IR spectra from C-D rich regions of a Gm larva fed 72h with $C_{16}D_{34}$ oil measured with a confocal microscope coupled to the synchrotron radiation source; the C-D peaks at 2197 and

2098 cm^{-1} reached absorbance values one order of magnitude higher than those measured with the hyperspectral imaging microscope, increasing measurement sensitivity and accuracy. F) Synchrotron-μFTIR spectra from PE microparticles in the gut of a PED4 fed larva.

Bio-degradation of PE films by dissected gut of Gm larvae

Since we could not detect any PE oxidation in the gut of the larvae, we then asked the question whether PE could be directly oxidised by the gut of the Gm larvae as reported by others[1]. This approach was criticized in Weber et al. (2017)[2] due to possible confusing factors such as contaminations and misinterpretations. Here we decided to use µFTIR hyperspectral imaging to study whether Gm gut content with or without microbiota could oxidize PE films. This approach allowed finely differentiating between regions where the PE film was oxidized and regions were the PE film was contaminated by residues of the larval gut. Guts from conventional and axenic Gm larvae were dissected and deposited on the surface of small pieces of an 8 µm thick HDPE (High density Polyethylene) plastic bag and incubated at 27°C in humid atmosphere for either 24 hours or 21 days (Figure S2A). The HDPE plastic bag pieces were then cleaned following a specifically developed protocol to eliminate gut residues (see the experimental section at the end).

IR hyperspectral images of the HDPE were recorded in transmission mode. PE was characterized by its methylene peak at 1465 cm^{-1} and PE oxidation by the carbonyl peak around 1735 cm^{-1}. The carbonyl/methylene peak area ratio was used to image the distribution of PE oxidation in the PE films in contact with the Gm larva gut. The presence of eventual gut residues was detected by measuring the presence of amide I (1650 cm^{-1}) and amide II (1545 cm^{-1}) bands from proteins. Clean pieces of HDPE bags (no contact with Gm guts) were used as control. Figure S2B shows representative spectra from the different plastic bags. The orange spectrum came from a control bag that was found to be naturally oxidised and showed a strong carbonyl peak with several shoulders evidencing multiple oxidation levels. It is noteworthy that the level of oxidation in that control bag was higher than in any of the treated bags by several orders of magnitude.

Even in the control bags we could detect weak carbonyl peaks at some discrete positions by using the hyperspectral images built from the carbonyl/methylene ratio (Figure S2C). This could arise from natural PE oxidation but also from the presence of other molecules such as polymer additives (plasticizers, anti-oxidants…).

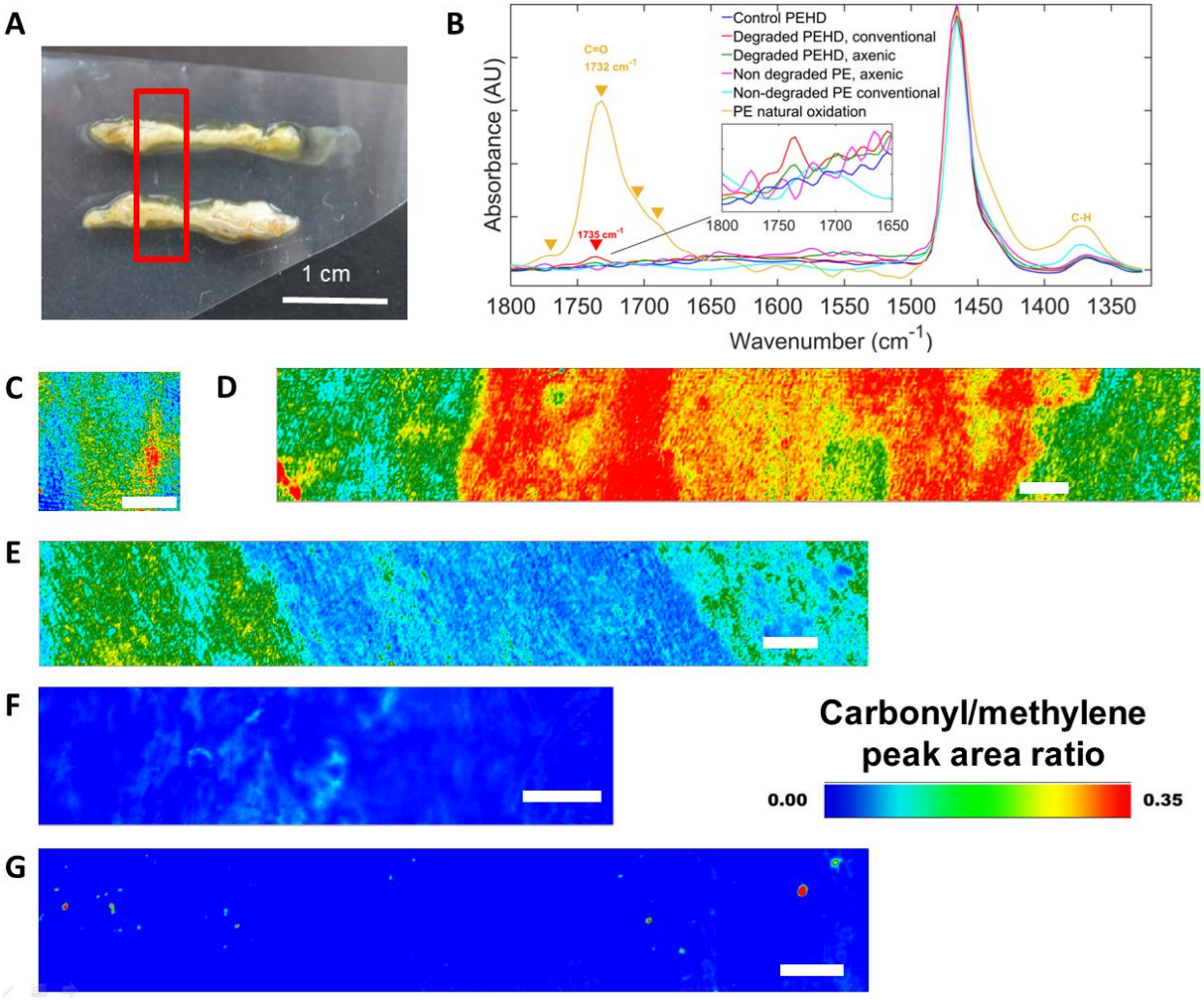

Figure S2. µFTIR imaging analysis of the degradation of HDPE films in contact with Gm larva gut. PE films were incubated at 27°C in humid atmosphere with the dissected gut of conventional or axenic larva deposited at the surface of the film, and spectral images of the PE films were recorded after 24 h or 21 days of contact. Hyperspectral images were generated by plotting the carbonyl/methylene (C=O/C-H) peak area ratio using the C=O peak at 1735 cm^{-1} and the C-H peak at 1370 cm^{-1} after water vapour subtraction (when necessary). Micrograph scale bar: 1 mm. A) Optical view of the dissected larva guts deposited on pieces of HDPE bags. B) Spectra of PE films in the 1300-1800 cm^{-1} range. Non-oxidized film (blue), ambient-air oxidized film (orange). The air oxidized film presents several peaks characteristic of various C=O moieties between 1650 and 1780 cm^{-1} and is dominated by the C=O from esters at 1732 cm^{-1}. Representative carbonyl/methylene spectral images of the PE films are shown in images C to F. A weak C=O peak at 1735 cm^{-1} characteristic of oxidation is observed in some but not

all of the films. C) control PE film showing regions with various oxidation levels. D) PE film degraded by the gut of a conventional Gm larva for 24h. E) PE film degraded by the gut of an axenic Gm larva for 24h. F) PE film degraded by the gut of a conventional Gm larva for 21 days. G) Image of the amide I band on a PE film degraded by the gut of an axenic Gm larva for 24h showing traces of protein and fat (green and red spots). The intensity scale of image G is 0.5-3.0 absorbance unit.

A weak C=O peak at 1735 cm^{-1} was detected in about two-third of the films exposed to the gut extract (Figure S2D-F) for both conventional and axenic samples but was undetectable in the other films. In the carbonyl/methylene peak area ratio images, the location of the carbonyl peak matches with the location of the gut on the film (Figure S2D, S2E). The carbonyl/methylene peak area ratio is reported in Supplementary Table 3). It was several times higher in the oxidized part of the plastic than in the non-oxidised regions or in the control film. However, the C=O peak was not detected or was lower than the control in some of the treated samples (conventional and axenic); and could barely be detected in the two samples incubated during 21 days (Figure S2F and Supplementary Table 3).

Supplementary Table 3. Oxidation level of PE by the gut of Gm larvae (Cv, conventional and Ax, axenic).

Larva #	Treatment	Duration (day)	HDPE oxidation CO:CH ratio	Change relative to control	Notes
T1	Control	-	0.54	-	Discrete oxidation
Cv I1	Gut + microbiota	3	0.64	1.18	Strongest
Cv I2	Gut + microbiota	3	3.10	5.70	$NaH(CO_3)$ contamination
Cv I3	Gut + microbiota	3	0.30	0.55	Discrete oxidized spots
Cv I4	Gut + microbiota	21	?	-	Strong water vapour
Cv I5	Gut + microbiota	21	0.03	0.04	Interference fringes
Ax I1	Gut	3	0.05	0.09	Contamination with unknown carbohydrate
Ax I2	Gut	3	?	-	$NaH(CO_3)$ contamination
Ax I3	Gut	3	0	-	No detectable oxidation

With the use of a rigorous cleaning protocol, over 90% of the film was clean of fats and proteins but we could detect the presence of gut residues at discrete positions of some PE films (Figure S2G), and also other contaminations originally present in the bags such as carbohydrates and $NaH(CO_3)$.

This experiment revealed several confusing factors such as water vapour signal, interference fringes, and residues from gut proteins and fats that obscured the detection of PE oxidation since the carbonyl peak was weak in all samples. The O-H bending mode from water vapour also strongly absorbs at 1735 cm^{-1}. Although the IR microscope was continuously purged with nitrogen, the water vapour signal was often found to be of the same amplitude as the C=O peak in the oxidized HDPE and a water vapour subtraction step had to be performed to confirm whether any oxidation was detected. Furthermore, even after thorough cleaning procedure was applied to HDPE bags to remove the gut tissue, discrete spots with residual proteins and lipids were detected in some samples. The C=O stretching mode from esterified lipids absorb at 1740 cm^{-1}. PE samples that are not correctly cleaned will be contaminated by fats whose stronger carbonyl band will totally obscure the weak carbonyl band from oxidized PE. These confusing factors limited both the sensitivity and specificity of the protocol. Although our findings suggested that PE might be weakly oxidised by the extracts of Gm larva gut and that the carbonyl/methylene ratio was stronger in the conventional than in the axenic cases, oxidation signals were weak and barely above detection threshold. We thus cannot firmly conclude about the implication of the gut microbiota in the oxidation of the PE.

Material and methods

Gm gut dissection protocol: 6 conventional and 6 axenically reared larvae were dissected, the larvae were cut longitudinally from the ventral side, and the skin spread with pins, the fat body

tissues were removed, and the digestive tube was removed from the mouth to the anus. The digestive tubes were opened longitudinally so that the inside of the gut could be placed in contact with the PE films.

HDPE plastic bags were cut in 3 cm by 7 cm triangular pieces. The HDPE pieces were sterilized on each side by UV light at 254 nm for 10 min. Two complete digestive tubes were placed on each of the HDPE pieces, then deposited in 6 Petri dishes and incubated at 27°C in humid atmosphere for either 24 hours or 21 days.

After the incubation of the digestive tubes on the HDPE, the pieces were cleaned by a specifically developed protocol: rinsed and shaken in 2 ml of water, followed by another bath in 5 ml of water at 60 ° C, then rinsed in 50% ethanol at 60°C to remove fat, then washed 5 times for 5 minutes in NaOH 1M at room temperature in an ultrasonic bath, and finally rinsed in distilled water and dried at room temperature.

Alteration of PED4 in larval frass

We wanted to assess if PED4 was altered during its transit through the larval gut. To do so we sought to observed changes in the CD_2/CD_3 ratio in PED4 particles measured in larval frass. The infrared spectra of the larval frass fed with PE or PED4 were measured by the ATR-FTIR technique. The presence of PED4 particles in the frass was evidenced by the presence of the C-D peaks between 2100 and 2200 cm^{-1}. The PED4 signal was low (0.02-0.008 A.U. at 2191 cm^{-1} for the stronger CD_2 peak). Frass from larva fed with normal PE did not present any C-D peaks. Due to the strong overlap of the CD_2 and CD_3 peaks, we used curve-fitting to extract the peak parameters. The C-D region of the spectra was fitted by 5 Gaussian/Lorentzian curves: 2191 (ν_{as} CD_2), 2175 (HCD), 2156 (ν_{as} Fermi resonance), 2086 (ν_s CD_2), 2066 (ν_s CD_3) cm^{-1}. The CD_2/CD_3 peak height ratio (RCD) was used to evidence PED4 oxidation and was computed for both the symmetric (ν_s) stretching vibrations. The antisymmetric CD_3 stretching vibrations between 2200 and 2250 cm^{-1} were barely noticeable.

Experimental

Larval frass were collected and stored at -80°C until measurement. Spectra were measured with a Perkin Elmer Spectrum 100 spectrometer (Perkin-Elmer, Les Ulis, France) equipped with a one millimetre diamond ATR crystal and DTGS detector. The spectra were recorded at 4 cm^{-1} resolution between 400 and 4000 cm^{-1} with 16-32 co-added scans.

The spectra were fitted with the Peak Resolve module of Omnic 9.2 software. The 1970-2370 cm^{-1} region containing 208 points was fitted with 5 Gaussian/Lorentzian peaks for a total of 35 free parameters (position, width, intensity, Gaussian factor; baseline).

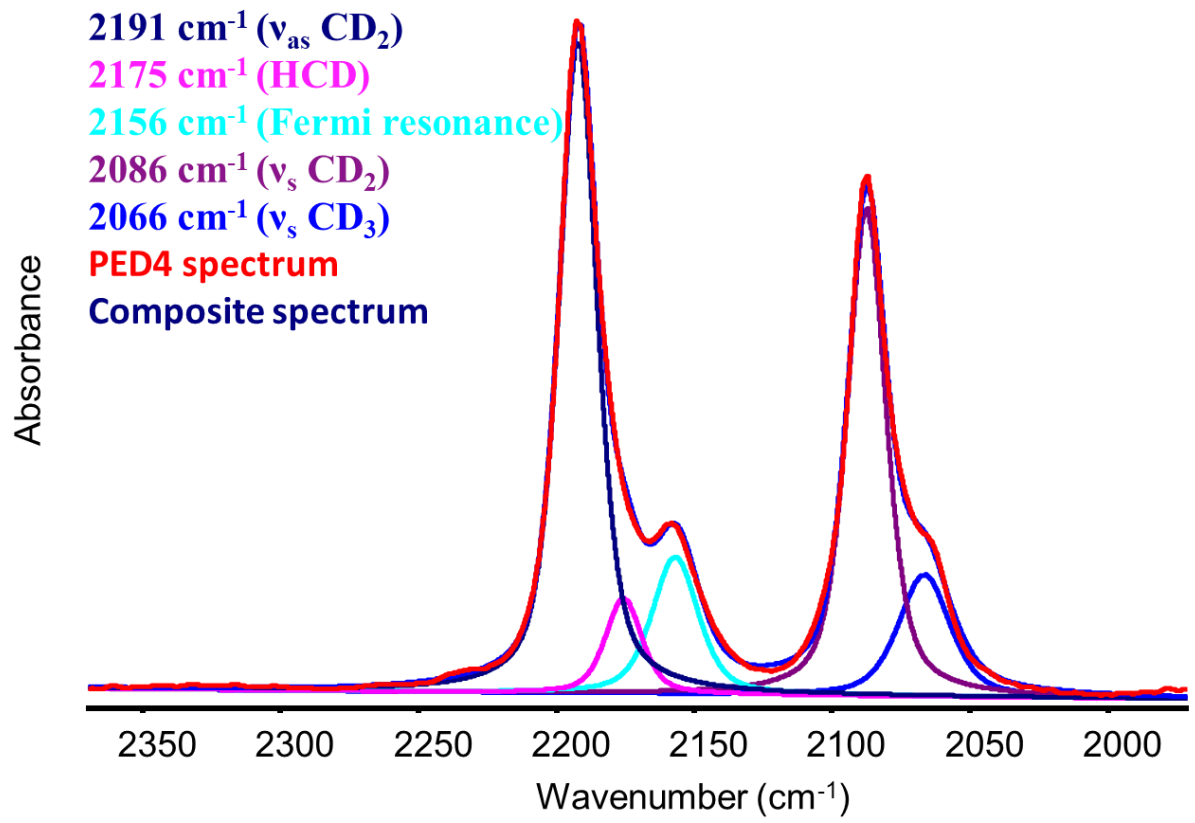

Figure S3. Curve-fitting of the C-D region of PED4 measured in the frass of a conventional larva. The PED4 peaks were fitted by components at 2191 (ν_{as} CD_2), 2175 (HCD), 2156 (Fermi Resonance), 2086 (ν_s CD_2), 2066 (ν_s CD_3) cm^{-1}. The computed spectrum (dark blue) matches the original PED4 spectrum (red).

Control PED4 films measured by the same method had a RCD of 4.46±0.29. The PED4 in frass had a lower RCD of 3.62±0.29, a 19% difference. Although the values were not found statistically different by a t-test (presumably a statistical oddity due to the low number of control PED4 films), we suggest that the change in CD_2/CD_3 peak height ratio indicated that the PED4 found in the frass had somewhat been processed and its aliphatic chain length may have been reduced.

Analysis of Gm larvae microbiota by 16S rRNA sequencing

Experimental

To analyse the microbiota of *Galleria mellonella*, L6 larvae (average weight 200 mg) were sampled from the Jouy-en-Josas rearing (fed with pollen and beeswax) and left without food for 24 h at 27°C. Frass were collected, larvae were dissected and the whole guts were removed and stored at -80 °C as were the feces.

DNA extraction from Galleria mellonella gut and feces: The weight of guts and feces used for DNA extraction were respectively 30-35 mg and 10-15 mg. The DNA extractions were carried out with the "Powerlyser PowerSoil DNA isolation kit" (MO BIO laboratories, inc.) according to the manufacturer's instruction with slight modifications as follows. After the addition of solution C1, samples were incubated at 65°C with stirring at 900 rpm for 10 min and high speed centrifugation for 1 min at 20,000 G was performed after the homogenization step, using FastPrep 24 instrument (MP Biomedicals) at 4m/s twice for 40s with a 5 min break at 4°C between each run. The DNAs were quantified with a Nanodrop 2000 (Thermo Scientific). The DNA concentrations were between 30 and 40 ng/µL per gut and the ratios 260:280 nm and 260:230 nm were >1.8. For the feces the ratios were >1.8 and the concentration was between 15 and 20 ng/µL.

Amplicon libraries, Illumina Miseq sequencing and analysis:

Amplicon libraries were constructed following two rounds of PCR amplification. The first amplification of the~450-bpV3- V4 hypervariable regions of the bacterial 16S rRNA gene was performed with the primers V3F (5'-ACGGRAGGCWGCAG- 3') and V4R (5'-TACCAGGGTATCTAATCCT-3') as described in Poirier et al.[21] Amplicon size, quality and quantity were checked on a DNA 1000 chip (Agilent Technologies, Paris, France). The purification and quantification of the second Illumina MiSeq PCR was made by Genotoul

(Toulouse-France) as well as the Illumina Miseq sequencing. Quality filtering, definition of OTUs and taxonomic assignment was made by Stephane Chaillou (INRAE-MICALIS, FME team) using FROGS pipeline (Find Rapidly OTU with Galaxy Solution).

Results:

The bacterial microbiota of the G. *mellonella* population analyzed by 16S rDNA sequencing, showed a composition largely dominated by two Enterococcus species, E. *mundtti* and E. *gallinarum*. Indeed the two species represent 98 % of the reads, in both feces and whole dissected guts (figure S4). In the remaining 2 % of the reads a few other species were found: other gram positive firmicutes as *Enterococcus faecalis* and *Lactobacillus kunkeei*, and the gram negative *Enterobacter arsenophonus*. This indicates that the here studied *Galleria mellonella* colony is not very riche in bacterial diversity, and it is similar to the study of Lou et & al.(2020).

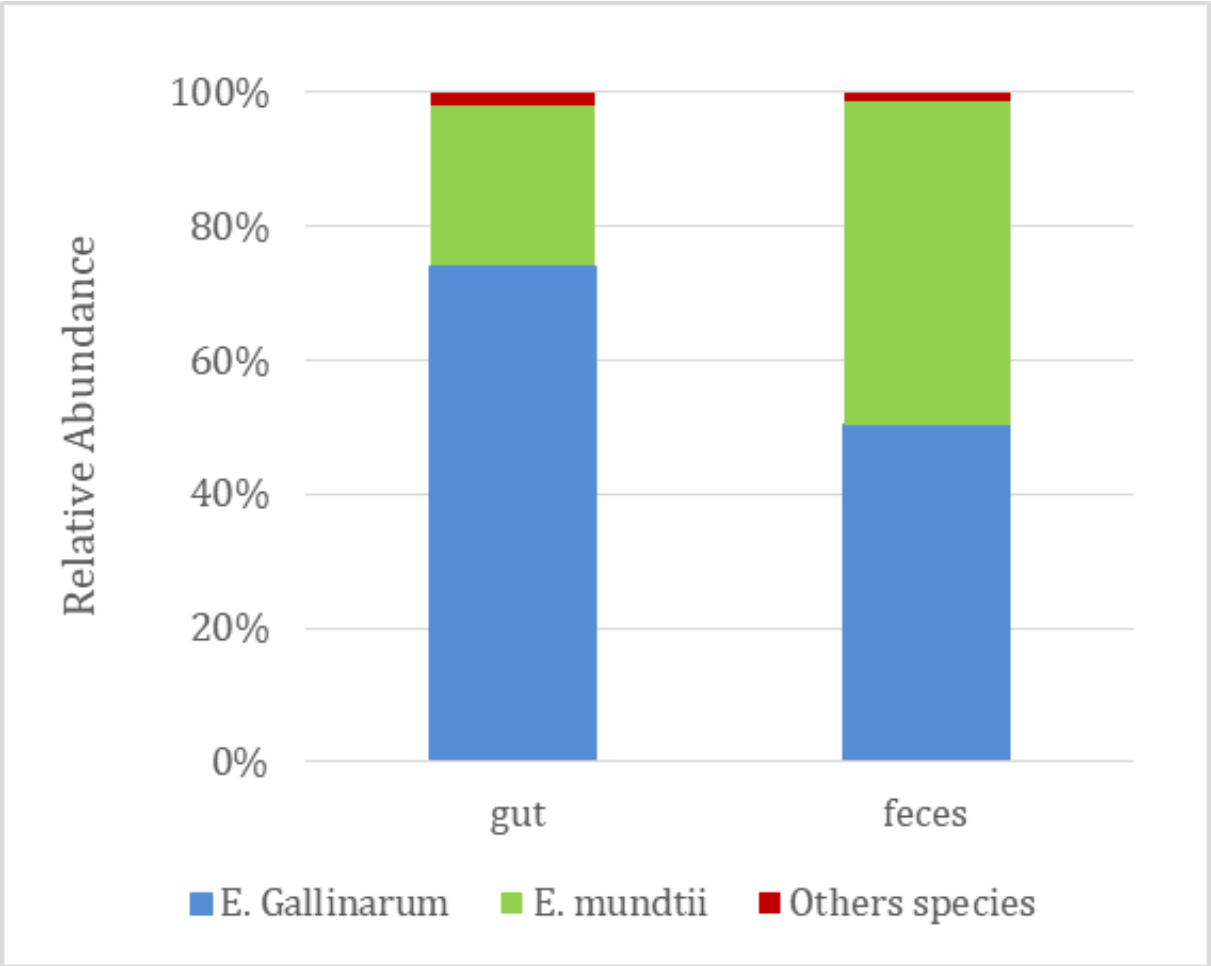

Figure S4: Relative abundance of bacterial species. Abundances following reads from MiSeq sequencing of 16S rDNA obtained from dissected guts (three replicates) and feces (six replicates) from *Galleria mellonella* larvae. 100% is the total number of reads.

Evaluation of PE crystallinity by FTIR spectroscopy

FTIR spectroscopy was used to evaluate the crystallinity of the polyethylene samples used in this study. Polymers present peaks that are specific of the amorphous and crystalline phases[3]. The relative intensities of these peaks can be used to evaluate the crystalline index of the polymer. The method has been developed for polyethyene in ATR measurement mode[4] and in

transmission[5] and shown to correlate well with calorimetric (DSC) estimation[5,6]. In this work we used the transmission method of Hagemann et al.[5] (to get better SNR) with modifications for the deuterated PE. We used the CH_2 rocking peaks between 700 and 740 cm^{-1} in order to estimate the crystallinity of normal PE and the CD_2 rocking peaks at 518 and 523 cm^{-1} for the crystallinity of the deuterated PED4.

Experimental

Pieces of HDPE and LDPE bags were cut and mounted on suitable sample holders then measured in transmission in the compartment of a Nicolet 5700 spectrometer with a thermal infrared source and DTGS detector. The mid-infrared spectra were measured between 400 and 4000 cm^{-1} at 2 cm^{-1} spectral resolution, and 64 to 128 scans were coadded. For each film, 3 measurements were recorded at different positions in the film and an average spectrum was computed and analysed.

Since the peaks are close and overlap, curve-fitting in the Omnic software was used to find peak intensities. Peaks were fitted by Gaussian-Lorentzian (GL) functions and fitted by a Levene-Marquardt algorithm. The PE spectra were fitted between 740 and 700 cm^{-1} while the PED4 spectrum was fitted between 540 and 500 cm^{-1}. Three GL peaks with 5 cm^{-1} initial FWHM were used.

The crystallinity index X_c was estimated for PE with the following formula:

$$X_c = \frac{I_{719}+I_{730}}{I_{719}+I_{730}+1.23*I_{721}}$$

were I_x are the intensities at wavenumbers 719, 730 and 721 cm^{-1}.

The crystallinity index X_c was estimated for PED4 with the following formula:

$$X_c = \frac{I_{519}+I_{526}}{I_{517}+I_{526}+1.23*I_{521}}$$

were I_x are the intensities at wavenumbers 519, 517, 526 and 521 cm^{-1}.

Results

In this work, the CH_2 rocking peaks for crystalline PE were found at 719.5 and 730 cm^{-1}, the CH_2 rocking peak for amorphous PE was found between 721 and 723 cm^{-1} depending on sample. The graphical output of the curve-fitting procedure for PEHD is given in Figure S5.

The HDPE bags were found to have higher X_c (0.83) than LDPE bags (X_c of 0.68).

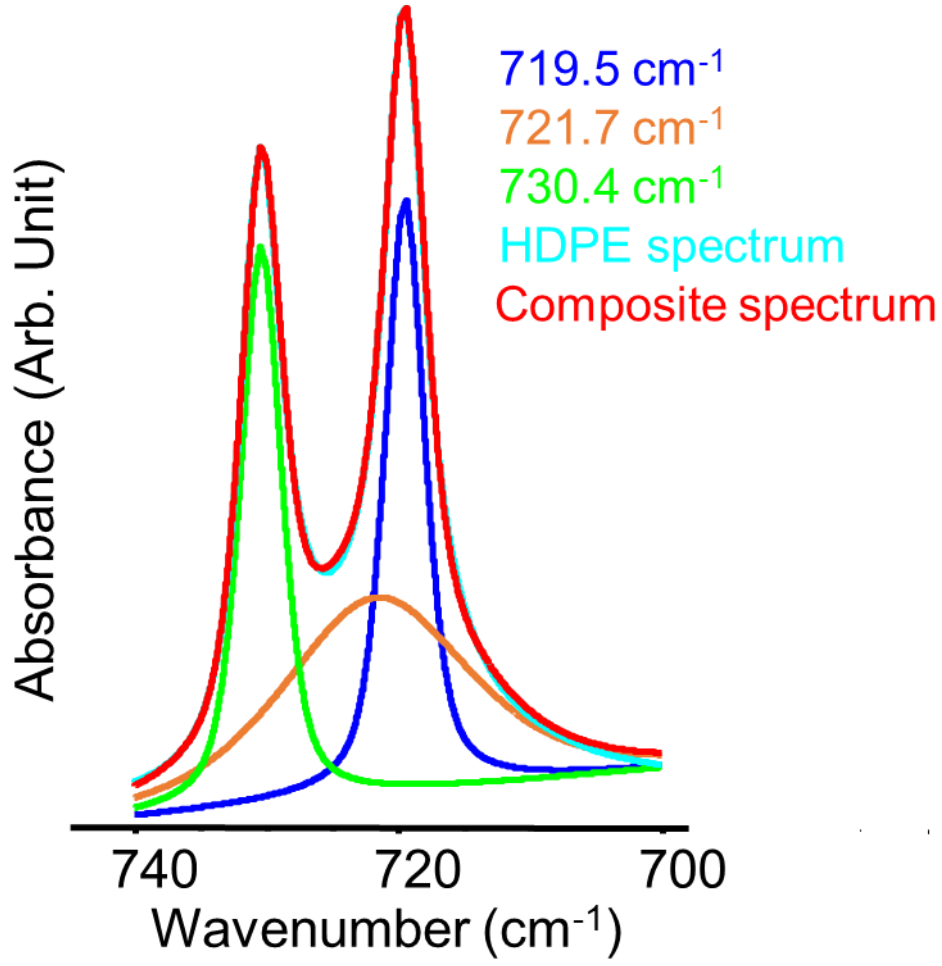

Figure S5. Result of the curve-fitting of HDPE spectrum between 700 and 740 cm^{-1}. Three GL

peaks are fit to the HDPE spectrum: crystalline PE peaks at 719.5 and 730.4 cm^{-1}, and the amorphous PE CH_2 rocking peak at 721.7 cm^{-1}. The computed spectrum (red) matches the actual spectrum (turquoise).

The PED4 crystallinity was estimated at 0.91 which is in the expected crystallinity range for HDPE (0.6-0.9).

Molecular weights and weight distributions of PE used in this study.

The molecular weights and molecular weight distributions were determined by High-Temperature Gel Permeation Chromatography (HT-GPC) by the Peakexpert company (Tours, France). Briefly, HT-GPC was performed at 150°C in stabilized trichlorobenzene on Agilent Mixed-B columns. Columns were calibrated with narrow MW distribution polystyrene references. The molecular weight distributions of the three PE samples (LDPE bags, HDPE bags and PED4) are shown in supplementary data (figure S6).

LDPE had a MW distribution centred at ~175k Da, HDPE at 150 kDa with a shoulder at 300 kDa and PED4 at 300 kDa.

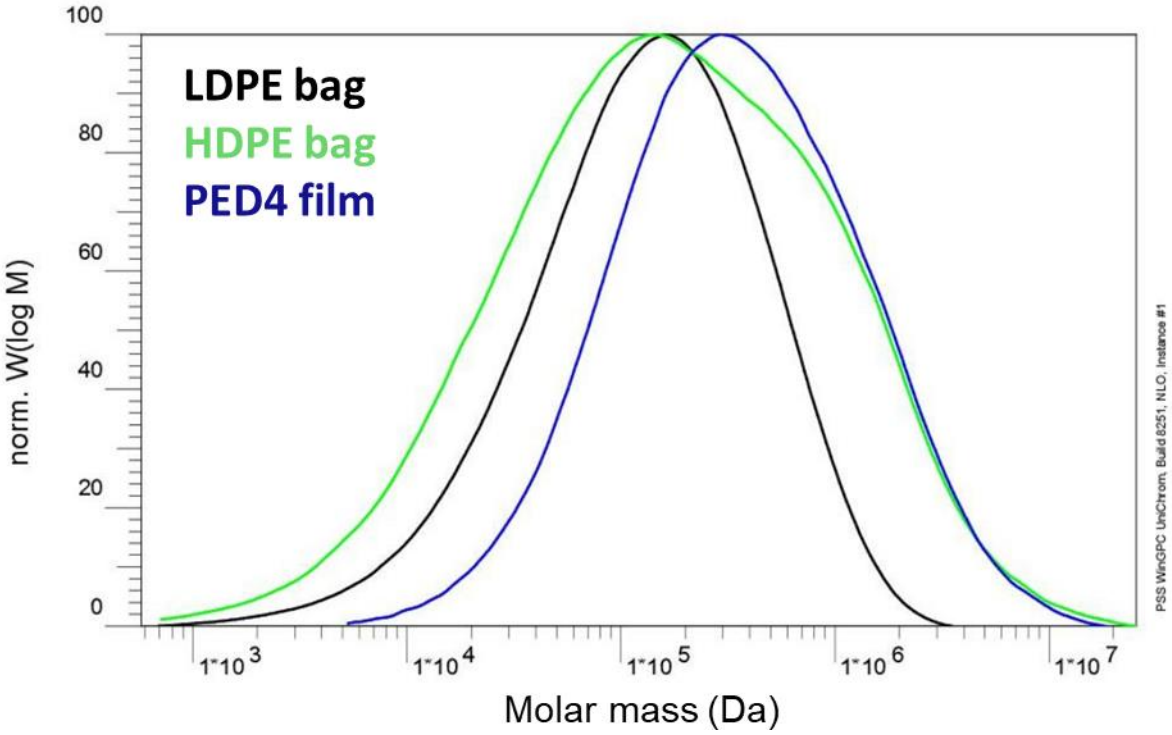

Figure S6: Normalized molecular weight distribution of the PE samples used in this study measured by HT-GPC.