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Oral exposure to polyethylene microplastics alters gut morphology, immune response, and microbiota composition in mice

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1 **Title page**

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3 Oral exposure to polyethylene microplastics alters gut morphology, immune response, and microbiota
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26 **Abstract**

27 The ubiquitous and growing presence of microplastics (MPs) in all compartments of the environment
28 raises concerns about their possible harmful effects on human health. Human exposure to MPs occurs
29 largely through ingestion. Polyethylene (PE) is widely employed for reusable bags and food packaging
30 and found to be present in drinking water and food. It is also one of the major polymers detected in
31 human stool. The aim of this study was to characterize the effects of intestinal exposure to PE MPs on
32 gut homeostasis. Mice were orally exposed for 6 weeks to PE microbeads of 2 different sizes, 36 and
33 116 μm , that correspond to those found in human stool. They were administrated either individually or
34 as a mixture at a dose of 100 $\mu\text{g/g}$ of food. Both PE microbead sizes were detected in mouse stool.
35 Different parameters related to major intestinal functions were compared between control mice, mice
36 exposed to each type of microbead, or co-exposed to the 2 types of microbeads. Intestinal disturbances
37 were observed after individual exposure to each size of PE microbead, and the most marked deleterious
38 effects were found in co-exposed mice. At the histomorphological level, crypt depth was increased
39 throughout the intestinal tissues. Significant variations of gene expression related to epithelial,
40 permeability, and inflammatory biomarkers were quantified. Defective recruitment of some intestinal
41 immune cells was observed from the proximal portion of the small intestine to the colon. Several
42 bacterial taxa at the order level were found to be affected by exposure to the MPs by metagenomic
43 analysis of cecal microbiota. These results show that ingestion of PE microbeads induces significant
44 alterations of crucial intestinal markers in mice and underscores the need to further study the health
45 impact of MP exposure in humans.

46

47 **Keywords**

48 Microplastics. Polyethylene. Mice. Intestinal. Inflammation. Microbiota.

49

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54

55 **Introduction**

56 Annual global plastic production has continuously risen since the 1960s and has reached about 368
57 million tons in 2019 (Plastics Europe, 2021). The total amount of plastic resins and fibers manufactured
58 from 1950 through 2015 is 7800 megatons (Geyer et al., 2017). Poor management in handling of plastic
59 waste has led to a tremendous increase in environmental dumping and ubiquitous spreading of
60 microplastic (MP) contamination (Jambeck et al., 2015; Rillig et al., 2021). MPs are defined as small
61 plastic particles less than 5 mm in size. MPs are purposefully manufactured for various applications,
62 such as exfoliants (microbeads) in personal care products (Wright and Kelly, 2017). This material, along
63 with plastic microfibers from machine-washed clothing, is directly released into the environment
64 through municipal effluent (Wright and Kelly, 2017). MPs present in the environment may also result
65 from fragmentation of larger plastic debris. MPs are detected in freshwater sources as well as tap and
66 bottled water (Danopoulos et al., 2020). They have also been detected in seafood (fish and shellfish),
67 salt, beer, honey, sugar, packaged meats, vegetables, and fruits (Hirt and Body-Malapel, 2020;
68 Kedzierski et al., 2020; Oliveri Conti et al., 2020).

69 Humans can be exposed to MPs via dermal and inhalational routes, but the risk of exposure by ingestion
70 is of special concern. No epidemiological study has established MP exposure as a causative risk factor
71 for intestinal diseases. However, early experimental studies performed mostly in aquatic organisms
72 showed that exposure to MPs lead to oxidative and inflammatory effects in the intestine and disruption
73 of gut epithelial permeability (Hirt and Body-Malapel, 2020). Regarding polystyrene MP, oxidative
74 stress was described in skeletal muscle, testes, ovaries and liver after oral exposure in mice (Deng et al.,

75 2017; Li et al., 2021; Shengchen et al., 2021; Wei et al., 2022; Xie et al., 2020). Several studies have
76 shown that polystyrene (PE) MP exposure lead to negative effects on the gut (Jin et al., 2019; Liang et
77 al., 2021; Lu et al., 2018; Luo et al., 2019). For example, a recent study showed that ingestion of PE
78 MPs in mice led to disturbances of gut and serum inflammatory parameters and significant modifications
79 of the intestinal microbiome (B. Li et al., 2020). This may have relevance to societal health since the
80 presence of PE has been detected in human stool (Schwabl et al., 2019; Zhang et al., 2021).

81 Globally, PE is the most highly produced synthetic, petroleum-based plastic material (Plastics Europe,
82 2021). PE is extensively used for the manufacture of disposable containers such as bottles and bags
83 (Bardají et al., 2020). Since 1938, PE has widely been applied as a plastic mulch in agriculture (Wang
84 et al., 2019). PE microbeads are also added to cosmetic products, although many countries have banned
85 the sale of rinse-off cosmetics containing MPs (Hunt et al., 2021). As a consequence of this expansive
86 use, inappropriate waste, and insufficient recycling, and also because PE is one of the most resistant
87 polymers to biodegradation, there is a massive accumulation of PE in the environment (Montazer et al.,
88 2020). For example, PE was the most highly abundant plastic polymer found in an Italian river,
89 accounting for 40.5% of total polymers (Munari et al., 2021). PE and polypropylene are the most
90 frequently detected plastic polymers in freshwater and tap water, and the second highest polymer
91 components of MPs in atmospheric fallout (Hirt and Body-Malapel, 2020).

92 In addition to the large quantities of PE which are accumulating in the environment, PE is also detected
93 in food. For instance, PE was the second most frequent polymer found in edible tissues of shellfish sold
94 for human consumption (Daniel et al., 2021). The widespread use of PE in food packaging for
95 preservation and easy handling purposes during transportation and storage also leads to migration from
96 food packaging to food (Katsara et al., 2021). Consequently, contamination of the environment and food
97 inevitably results in human exposure. Indeed, it has shown that PE, along with polypropylene,
98 polyethylene terephthalate, and polystyrene, are the predominant polymers found in human stool
99 (Schwabl et al., 2019). Moreover, Zhang et al. detected PE in 50% of stool samples collected from
100 Chinese students (Zhang et al., 2021). Noteworthy, the size of MPs detected in human feces ranged from
101 20 to 800 μm . More recently, human placenta and meconium samples were screened positive for PE

102 (Braun et al., 2021). Therefore, multiple lines of evidence show that intestinal tissues are in contact with
103 PE MPs.

104 To further assess the impact of oral PE exposure, mice were exposed for 6 weeks to food spiked with
105 commercial PE microbeads of 2 different average sizes (36 and 116 μm). Mouse stool was analyzed for
106 the presence of the microbeads. Histological analysis and quantification of major parameters of intestinal
107 epithelium were performed to evaluate gut effects. Intestinal immune response was assessed by
108 measurement of cytokine levels and immunophenotyping in small intestine and colon. Finally,
109 composition of the gut microbiome was analyzed by 16S rRNA pyrosequencing.

110 **1. Material and methods**

111 **1.1. Particles and chemicals**

112 PE microbeads were acquired from Cospheric (Santa Barbara, USA). Two categories of microbeads
113 were selected, namely red fluorescent beads (Item# UVPMS-BR-1.090 10-45 μm) and green fluorescent
114 beads (Item# UVPMS-BG-1.00 106-125 μm). The claimed diameter sizes range from 10 to 45 μm for
115 the red beads (RB) and 106 to 125 μm for the green beads (GB).

116 For chemicals, 10% (w/w) KOH was purchased from Chimie Plus (Vitry-sur-Seine, France), ultra-pure
117 water was acquired from Carlo Erba (Val-de-Reuil, France), and absolute ethanol from VWR (Fontenay-
118 sous-Bois, France).

119 **1.2. Size characterization**

120 An Olympus SZX-16 stereomicroscope equipped with a SDFPLAPO PF 1x/0.15 objective and a UC90
121 camera was used to capture images by trans-illumination. A magnification of 11.5x was employed for
122 RB and 5x for GB. Microbeads were manually measured using a 3-point circle tool with OlyVIA
123 software (ver. 3.1, build 19668). A total of 312 particles were measured for each category of beads on
124 respectively 5 and 4 fields for RB and GB. Descriptive statistical data were computed using Microsoft
125 Excel.

1.3. Identification of polymeric composition

In order to ascertain the polymeric composition, a microbead of each category was analyzed using a Spotlight™ 400 Fourier-transform infrared (FTIR) spectrometer equipped with a MCT detector coupled to a Spectrum 3 MIR spectrometer. The automatic micro Attenuated Total Reflection (μ ATR) module was used to acquire spectra. All the recorded spectra were obtained in the transmittance mode in the 4000–600 cm^{-1} region with 2 cm^{-1} resolution and 5 accumulations. Spectra were compared for the 3500–1200 cm^{-1} area to custom reference spectral libraries. Each identification was considered as valid from a score of >0.7 .

1.4. Mice and experimental design

All animal procedures were conducted in accordance with the institutional guidelines approved by the institutional Animal Care and Ethical Use Committee of the University of Lille (committee no.75; authorization no. CEEA2017031312157794). C57BL/6 mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and housed under standard conditions. Female mice from 7 to 12 weeks old were used in the study. Three independent experiments were performed. Age- and sex-matched mice were used in all experiments. Mice were randomly assigned to 4 experimental groups: 1) control group, receiving control food (final $n=9$); 2) RB group, receiving food spiked with 100 μg of red PE microbeads/g of food (final $n=10$); 3) GB group, receiving food spiked with 100 μg of green PE microbeads/g of food (final $n=10$); and 4) RB+GB group, receiving food spiked with 100 μg of red PE microbeads and 100 μg of green PE microbeads/g of food (final $n=10$). The intoxication lasted 6 weeks. At necropsy, proximal and distal small intestine, cecum, and colon were sampled.

1.5. Extraction from mouse feces

As a first attempt, mouse feces exposed to both types of microbeads were gently triturated using a tweezer into the bottom of a Petri dish filled with water and observed using an Olympus SZX-16 stereomicroscope with a SDFPLAPO PF 1.6x/0.15 objective.

After feasibility tests, extraction of microbeads from mouse feces was carried out by adapting the method of Dehaut et al., 2016, following the temperature decrease proposed by Treilles et al., 2020

152 (Dehaut et al., 2016; Treilles et al., 2020). Feces were analyzed individually. For each of the 4
153 conditions, n=8 feces were weighed on a 0.0001 g sensitivity Sartorius ME215-P analytical balance
154 (Dourdan, France) and placed in a glass beaker. The average mass of feces was 48.1 ± 29.3 mg. After
155 weighing, 10 mL of 10% KOH (m/m) was poured in the beakers and a 2.5 cm magnetic stirrer was
156 added. For digestion, beakers were placed on two multiple stirrers (MIXDrive 6 HT; 2mag, Munich,
157 Germany) with an agitation of 200 rpm for 4 h in a Binder BD 240 incubator (Tüttlingen, Germany)
158 with a temperature of $39.9 \pm 1.3^\circ\text{C}$. Once digested, solutions were filtered with a vacuum system onto a
159 90 mm GF/A 1.6 μm glass fiber filter set between a VWR 1100 mL funnel and a sintered glass filter
160 holder (Fontenay-sous-Bois, France). The rinsing protocol was carefully performed to increase recovery
161 of the particles on the filter. This protocol consisted of a three-step rinsing using ultra-pure water / 70%
162 (v/v) ethanol / ultra-pure water that was applied on the empty beaker, the empty funnel attached to the
163 filter holder, and a final rinse of the contact area between the funnel and the filter holder.

164 For a positive control (PC), 10 samples with particles were processed in the same conditions for each
165 batch of digestion. This PC was composed of 10 GB for mice fed with GB, 10 RB for those fed with
166 RB, and 5 GB and 5 RB for mice fed with the mixture of microbeads.

167 **1.6. Histological analysis**

168 At necropsy, tissues were fixed in 4% paraformaldehyde overnight, processed, and embedded in paraffin
169 wax by standard techniques. Serial histological sections of 4 μm thickness were cut, deparaffinized,
170 rehydrated, and stained with Alcian blue and periodic acid-Schiff (AB-PAS). Epithelial area in the
171 colon, villus height, and crypt depth in the proximal and distal small intestine were measured using
172 ImageJ software. For this study, at least 100 well-oriented mucosa, villi, and crypts were measured in at
173 least 5 individual mice from each group. AB-PAS-positive cells were counted under a light microscope
174 (Leica DM5500B).

175 **1.7. Real-time quantitative polymerase chain reaction (PCR)**

176 Small intestinal and colonic tissue samples were homogenized with ceramic beads using Precellys
177 Lysing Equipment (Bertin Technologies, Montigny-le-Bretonneux, France). Total RNA was extracted

178 with the NucleoSpin RNA kit (Macherey-Nagel, Hoerd, France). Transcript levels of genes were
179 quantified with the StepOne™ Real-Time PCR system using a SYBR Green PCR master mix (Thermo
180 Fisher Scientific, Villebon-sur-Yvette, France). The primer sequences were designed using Primer
181 Express 3 (Thermo Fisher Scientific) and are available upon request. Melting curve analyses were
182 performed for each sample and gene to confirm the specificity of the amplification. The relative
183 expression of each target gene was normalized to the relative expression of the *Polr2a* gene.
184 Quantification of target gene expression was based on the comparative cycle threshold (Ct) value. The
185 fold changes in the target genes were analyzed by the $2^{-\Delta\Delta C_t}$ method.

186 **1.8. Cell isolation and flow cytometry**

187 At necropsy, the colon and small intestine were harvested and cleaned of fat residue and Peyer's patches
188 for the small intestine. Tissues were open longitudinally, cut into small pieces, and rinsed in cold PBS
189 containing 2% SVF. Pieces were then incubated in predigestion buffer containing EDTA and DTT
190 (Merck KGaA, Darmstadt, Germany) for 30 min at 37°C under agitation. Pieces were then filtered
191 through a 100 µm cell strainer and incubated in digestion solution containing collagenase type 1 (Merck
192 KGaA, Darmstadt, Germany) for 45 min at 37°C under agitation. Cell solution was then passed through
193 a 100 µm cell strainer. Supernatants from the predigestion and digestion steps were then combined and
194 centrifuged. The pellet was resuspended in 44% Percoll (GE Healthcare, Buc, France) and carefully
195 overlaid on a 67% Percoll solution. The Percoll gradient was centrifuged for 20 min and immune cells
196 were recovered from the white ring visible at the interphase of the 2 Percoll solutions. Cells were
197 resuspended in PBS containing a marker of cell viability (Fixable Viability Stain 780; BD Biosciences,
198 Le Pont-de-Claix, France) for 10 min to discriminate viable from non-viable cells in FACS analysis.
199 Cells were then incubated with Fc Block (anti-CD16/CD32; BD Biosciences) for 10 min and then with
200 antibodies in FACS buffer (Brilliant Stain Buffer; BD Biosciences) for 30 min. Antibodies against
201 CD11c (V450; BD Biosciences), CD45 (BV570; Biolegend, San Diego, USA), LY-6G (BV605; BD
202 Biosciences), CD64 (PE; BD Biosciences), I-A/I-E (PE/Dazzle 594; Biolegend), CD11b (BB700; BD
203 Biosciences), CX3CR1 (PE/Cy7; Biolegend), Ly-6C (APC; BD Biosciences), CD4 (PE-Cy7; BD
204 Biosciences), and CD8a (AF532; eBioscience, Thermo Fisher Scientific, Villebon-sur-Yvette, France)

205 were used along with the isotype control antibodies V450 Hamster IgG1 (BD Biosciences), BV570 Rat
206 IgG2b (Biolegend), BV605 Rat IgG2a (BD Biosciences), PE Mouse IgG1 (BD Biosciences), PE/Dazzle
207 594 Rat IgG2b (Biolegend), BB700 Rat IgG2a (BD Biosciences), PE/Cy7 Mouse IgG2a (Biolegend),
208 APC Rat IgM (BD biosciences), PE-Cy7 Rat IgG2a (BD Biosciences), and AF532 Rat IgG2a
209 (eBioscience). After washing, cells were analyzed by flow cytometry (Sony SP6800). The generated
210 data were analyzed using FlowJo software (ver. 10.7.1; TreeStar, Stanford, USA).

211 **1.9. Bacterial DNA extraction and Illumina MiSeq sequencing**

212 Genomic DNA was extracted from cecal content using the DNA stool kit (Macherey Nagel, Hoerd, t,
213 France). The quantity and purity of DNA (expressed as the ratio of absorbance at 260 and 280 nm) were
214 assessed using a NanoDrop® spectrophotometer (Ozyme, Saint-Cyr-l'Ecole, France). The sequencing
215 library was generated by amplifying the V3-V4 region of the bacterial 16S-rRNA gene using 16S rRNA
216 amplicon generation for MiSeq with the primers Bact-0341 (CCTACGGGNGGCWGCAG) and Bact-
217 0785 (GACTACHVGGGTATCTAATCC). Individual samples were barcoded, pooled to construct the
218 sequencing library, and sequenced using an Illumina MiSeq system (Illumina, San Diego, USA) to
219 generate paired-end 2x300 bp reads.

220 **1.10. Analysis of sequencing data**

221 Bioinformatic analyses were performed using the QIIME2 pipeline (ver. 2020.2) (Bolyen et al., 2019).
222 The Divisive Amplicon Denoising Algorithm 2 (DADA-2) plug-in in QIIME2 was used to filter,
223 dereplicate, identify chimeric sequences, and merge reads to obtain the set of Amplicon Sequence
224 Variants (ASVs) for each sample (Callahan et al., 2016). Then the representative sequences were picked
225 for each ASV. The classify-sklearn plug-in in QIIME2, with the SILVA database (ver. 132), was applied
226 to assign a taxonomic annotation to each representative ASV sequence. In the next step, ASVs identified
227 as eukaryotic contamination (3 ASVs; 12 reads) and external contamination, identified with the
228 decontam package (3 ASVs; 3119 reads), were filtered out (Davis et al., 2018). Diversity metrics (α and
229 β) were obtained with the QIIME2 core-metrics-phylogenetic plug-in, with p-sampling depth parameter
230 fixed to 13781 reads which corresponded to the total frequency that each sample should be rarefied to

231 prior to computing diversity metrics. This sampling depth allowed retention of >61% of reads and the
232 discarding of only one sample. Tests for differential relative abundance were performed with corncob
233 at the order, family, and genus levels (Martin et al., 2020).

234 **1.11. Statistics**

235 Results are expressed as mean \pm standard error of the mean (SEM). The statistical significance of
236 differences between experimental groups was calculated using the Mann-Whitney nonparametric U test
237 (GraphPad Prism software, USA). Statistical significance was defined as $p < 0.05$. For all experiments,
238 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, and **** $p < 0.001$ vs control group.

239 **2. Results**

240 **2.1. Characterization of microbeads**

241 For size characterization of RB, it was observed that the majority of beads, 94.2%, were included in the
242 size range defined by the supplier (10-45 μm) (Fig. 1A). The percentage was lower for GB, in which
243 only 83.7% of spheres were in the claimed size range (100-125 μm), and 14.1% of particles were lower
244 than 106 μm (Fig. 1B). The median size of spheres was 36 and 116 μm for RB and GB, respectively.
245 This indicates that the size distribution is rather shifted towards the largest particle sizes in the range for
246 each of the bead references. Overall, the size distributions of RB and GB were significantly different
247 (Fig. 1C, $p < 0.001$).

248 Based on observation, the large majority of particles were spherical single chimeric particles for both
249 RB and GB. Some minute polymeric debris, ca. 40, irregular shaped microparticles with size below 10
250 μm were also observed on the 5 fields of RB.

251 **2.2. Identification of polymeric composition**

252 Based on FITR spectra, the respective top scores obtained for GB and RB were 0.9593 and 0.95889,
253 matching with PE, without signal for other polymers, confirming that microbeads are composed of pure
254 PE polymer (Fig.1D, E)..

255

256 2.3. Study of ingested microbeads

257 2.3.1. Direct observation of mouse feces

258 During our first attempt to visualize the presence of both sizes of microbeads in mouse stool, both intact
259 and fragmented microbeads were observed (Fig. 1F).

260 2.3.2. Count and observation after alkaline hydrolysis

261 Regarding the PCs used during this experiment, different results were obtained depending on the type
262 of beads. Mostly, GB were recovered with acceptable results (Table 1). In one case, 2 beads proved to
263 be fragmented. RB were more difficult to be recovered.

264 **Table 1: Counts of intact and fragmented beads in the positive controls.**

	Counts*
Green beads	7/10
Red beads	7/10
Mixture	5/5 (GB)** 2/5 (RB)

* (GB) Green beads (RB) Red beads

** 2 out of the 5 beads were observed as fragments

265

266 For fecal analysis (Table 2), neither GB nor RB was observed in the control mice. For digested feces,
267 observed after exposure of mice to green, red, or a mixture of both types of beads, all samples contained
268 beads except for a single feces of mouse treated with GB.

269

270 **Table 2 : Count data of intact and fragmented beads observed in the samples (n=8) for the 4 exposure treatments.**

	*	Minimum	Maximum	Range	Mean	Std.Error of Mean
Green beads	I	0	5	5	2.9	0.69
	F	0	20	20	4.9	2.3
Red beads	I	12	135	123	64	15
	F	15	88	73	40	11
Mixture	IG	1	5	4	2.8	0.53
	IR	23	83	60	46	7.5
	FG	2	44	42	17	5.5
	FR	8	173	165	55	19

Controls	I	0	0	0	0	0
	F	0	0	0	0	0

271 * I: intact beads, F: fragmented beads, IG: intact green beads, IR: intact red beads, FG: fragmented green
 272 beads, FR: fragmented red beads.

273 The microbeads were present as both intact and fragmented particles but counts were variable depending
 274 on feces, and this heterogeneity was independent of the fecal mass (Table 3). Higher counts were
 275 recorded for RB, which was an expected result since food was supplemented with PE MPs based on
 276 mass quantities. There was a tendency to have a higher proportion of fragmented GB and intact RB in
 277 comparison with their respective counterparts.

278

279 **Table 3 : Data on oncentrations expressed as number per mg of feces for intact and fragmented beads observed in the**
 280 **samples (n=8) for the 4 exposure treatments.**

	*	Minimum	Maximum	Range	Mean	Std.Error of Mean
Green beads	I	0.0	0.2	0.2	0.1	0.033
	F	0.0	0.5	0.5	0.15	0.063
Red beads	I	0.2	2.9	2.7	1.6	0.29
	F	0.3	1.7	1.4	1.0	0.19
Mixture	IG	0.0	0.2	0.2	0.088	0.030
	IR	0.6	3.7	3.1	1.4	0.38
	FG	0.1	0.8	0.7	0.41	0.11
	FR	0.2	3.8	3.6	1.4	0.40
Controls	I	0.0	0.0	0.0	0.0	0.0
	F	0.0	0.0	0.0	0.0	0.0

281 * I: intact beads, F: fragmented beads, IG: intact green beads, IR: intact red beads, FG: fragmented green
 282 beads, FR: fragmented red beads.

283

284 **2.4. Effects of PE microbead exposure on proximal small intestine**

285 After 6 weeks of exposure, weight gain was similar between the 4 groups of mice (112.2±2.8% for CT,
 286 112.3±2.6% for RB, 111.5±1.7% for GB, and 112.6±2.6% for RB+GB, $p=0.9$ vs CT group). In order to
 287 determine if exposure to PE induces morphological alterations of the proximal small intestine,
 288 morphometric analyses of this tissue were performed. The measurement of the villus length did not show
 289 significant changes in any of the groups (Fig. 2A, B). The crypt depth was increased in the mice exposed
 290 to GB ($p=0.006$) and RB+GB ($p=0.002$) compared to control mice (Fig. 2A, B). Moreover, the
 291 villus/crypt ratio was decreased in the 2 groups of mice exposed to PE individually ($p=0.03$ for RB and
 292 $p=0.0006$ for GB) and in the group of mice exposed to the mixture of the 2 PE types ($p=0.0003$; Fig.

293 2C). The AB-PAS-positive area, i.e., the mucin-positive area, was lower in mice exposed to GB
294 ($p=0.007$) and in mice exposed to RB+GB ($p=0.0002$; Fig. 2A, D). The transcript levels of the major
295 secretory protein mucin-2 (*Muc2*) was significantly decreased in RB- and GB-exposed mice ($p=0.001$
296 and $p=0.04$, respectively; Fig. 2E). Expression levels of major markers of epithelial cell types were also
297 quantified. The absorptive epithelial cell marker villin-1 (*Vill*) was decreased in RB- and GB-exposed
298 mice ($p=0.001$ and $p=0.04$, respectively; Fig. 2E). The enteroendocrine cell marker chromogranin-A
299 (*Chga*) was downregulated in GB- ($p=0.001$) and RB+GB-exposed mice ($p=0.001$; Fig. 2E). The stem
300 cell marker leucine rich repeat containing G protein coupled receptor 5 (*Lgr5*) did not vary (Fig. 2E).
301 The levels of the tight junction-related genes occludin (*Ocln*) and junctional adhesion molecule A (*F11r*)
302 were not modified in PE-exposed mice (Fig. 2F). Assessment of the inflammatory status of the proximal
303 small intestine showed that the mRNA levels of tumor necrosis factor alpha (*Tnf*), interferon gamma
304 (*Ifng*), interleukin-6 (*Il6*), and interleukin-1 beta (*Il1b*) proinflammatory cytokines were not significantly
305 different between the PE-exposed and the control mice (Fig. 2G). Immunophenotyping showed
306 significant variations of the frequency of 4 immune populations: CD4⁺ T lymphocytes ($p=0.03$), CD8⁺
307 T lymphocytes ($p=0.03$), dendritic cells ($p=0.04$), and inflammatory monocytes ($p=0.004$) were
308 reduced in mice exposed to the mixture of the 2 PE microbeads (Fig. 2H).

309 **2.5. Effects of PE microbead exposure on distal small intestine**

310 For the distal small intestine, an increase of crypt depth was found in RB- ($p=0.01$) and RB+GB-exposed
311 mice ($p=0.003$; Fig. 3A, B). The villus/crypt ratio was significantly decreased in the 3 groups of PE-
312 exposed mice ($p=0.006$, $p=0.03$, and $p<0.0001$ for RB, GB, and RB+GB, respectively; Fig. 3C). The
313 mucin area was not significantly modified in any of the PE-exposed groups, as well as *Muc2* transcript
314 expression (Fig. 3A, D, E). In the distal small intestine, a significant increase of *Vill*, *Chga*, and *Lgr5*
315 was also measured in RB+GB-exposed mice compared to control mice ($p=0.003$ for the 3 targets; Fig.
316 3E). *Ocln* and *F11r* mRNA levels tended to decrease in the PE-exposed mice, with a significant decrease
317 of *Ocln* observed in the RB+GB-exposed mice compared to control mice ($p=0.04$; Fig. 3F).
318 Furthermore, increasing trends of *Tnf*, *Ifng* and *Il1b* were observed in the PE-exposed group, with a
319 significant rise of *Ifng* in GB-exposed mice compared to control mice ($p=0.03$; Fig. 3G). The levels of

320 *Il6* tended to decrease in PE-exposed mice, with a significant diminution observed in the mice exposed
321 to both PE sizes ($p=0.01$; Fig. 3G). The frequency of NK cells was increased in distal small intestine of
322 mice exposed to the mixture of PE MPs ($p=0.05$; Fig. 3H).

323 **2.6. Effects of PE microbead exposure on colon**

324 In colon, the mucosal surface area tended to increase in RB- and GB-exposed mice, and a significant
325 enhancement of the mucosal surface was observed in RB+GB-exposed mice compared to control mice
326 ($p=0.03$; Fig. 4A, B). The mucin-positive area was also significantly increased in the RB+GB-exposed
327 mice compared to control mice ($p=0.003$; Fig. 4A, C). Accordingly, *Muc2* mRNA levels were also
328 significantly upregulated between these 2 groups ($p=0.0003$; Fig. 4D). The expression of *Lgr5* was
329 enhanced in the GB group ($p=0.02$; Fig4D). The transcripts of *Vill* and *Chga* were upregulated in colon
330 of GB ($p=0.02$ and $p=0.004$, respectively) and RB+GB groups ($p=0.001$ for both genes; Fig. 4D). These
331 2 groups also presented a significant overexpression of tight junction-related genes *Ocln* and *F11r*
332 (respectively $p=0.001$, $p=0.002$ for GB group and $p=0.008$, $p=0.003$ for RB+GB group). For
333 inflammatory cytokines, an upregulation of *Tnf*, *Ifng*, *Il6*, and *Il1b* transcripts was observed in the colon
334 of mice exposed to the mixture of PE compared to control mice, although only *Ifng* and *Il6* reached a
335 significant level ($p=0.002$ for *Ifng*, $p=0.005$ for *Il6*; Fig. 4F). *Ifng* expression was induced by the
336 exposure to GB MPs ($p=0.046$). Furthermore, the relative abundance of polynuclear neutrophils was
337 higher in the GB-exposed mice ($p=0.02$), and the abundance of anti-inflammatory macrophages was
338 lower in both GB- and RB+GB-exposed mice ($p=0.05$; Fig. 4G).

339 **2.7. Effects of PE microbead exposure on microbiome composition**

340 To assess the impact of PE exposure on the mouse microbiome, V3-V4 amplicons of 16S rRNA genes
341 were sequenced in the cecal content. Exposure to PE did not significantly affect α diversity (Chao1
342 diversity index; Fig. 5A). The unweighted-UniFrac index showed a significant decrease of β diversity
343 in GB-exposed mice compared to control mice ($p=0.02$; Fig. 5B). Taxonomic assignment at the phylum
344 level of ASVs, with each color representing an individual bacterial phylum, is shown in Fig. 5C. As
345 expected, *Bacteroidetes* and *Firmicutes* represented the 2 predominant phyla. The effect of PE exposure

346 was assessed on the abundance of bacterial orders. Several bacteria showed significant changes in
347 relative abundance between the 3 PE-exposed groups and the control group (Fig. 5D). The GB-exposed
348 mice had more abundant Erysipelotrichaceae bacteria ($p=0.04$). Both RB- and GB-exposed mice had
349 reduced amounts of Verrucomicrobiales ($p=0.04$ for both groups). By contrast, the relative abundance
350 of Gastranaerophilales was heightened in these 2 PE-exposed groups ($p=0.03$ for RB- and $p=0.04$ for
351 GB-exposed mice). An increasing trend for Gastranaerophilales abundance was observed in the
352 RB+GB-exposed group ($p=0.06$). Lastly, the abundance of Rhodospirillales and Lactobacillales was
353 shown to be increased and decreased, respectively, in RB+GB-exposed mice compared to control mice
354 ($p=0.02$ for both orders).

355 **Discussion**

356 The aim of this study was to assess whether the presence of PE in intestinal lumen can induce host
357 disturbances. We studied the effects of oral exposure to PE of 2 different average sizes (36 and 116 μm),
358 which present about a 3-fold difference in diameter. These sizes were chosen because they reproduce
359 what has been detected in human feces (Zhang et al., 2021). The concentration of PE was 100 $\mu\text{g/g}$ of
360 food. Assuming the food intake of C57BL/6 mice is 5 g of food/30g body weight (bw)/day, the daily
361 intake of PE was approximately 16.66 mg PE/kg bw/day (Bachmanov et al., 2002). Using the usual
362 interspecies (animal to human) uncertainty factor of 10, this concentration can be extrapolated as 1.66
363 mg PE/kg bw/day (Dourson et al., 2021). Recently, human ingestion of MPs has been estimated between
364 0.1 to 5 g weekly, or 0.2 to 10.2 mg/kg bw/day, for a 70 kg adult (Senathirajah et al., 2021). Therefore,
365 the concentration of PE tested was chosen to reflect a realistic human ingestion range of MP. We also
366 tested MP exposure either individually or as a mixture in order to address the issue of potential additive,
367 inhibitory, or synergistic effects.

368 Our initial analyses confirmed that RB and GB were detected in mouse stool. In the experiment with
369 beads of the positive control, the recovery rate was close to 80% despite the care undertaken to rinse all
370 glassware used for the isolation process with both ultra-pure water and 70% (w/w) ethanol solution. This
371 incomplete recovery may be explained by a stickiness phenomenon resulting from interactions between

372 glass and the beads. Our analyses also showed a trend of microbead fragmentation in mouse stool. Based
373 on the direct observation of stool without digestion and beads of the positive control, it appears that
374 fragmentation of beads might have occurred during the ingestion and digestion by mice. A second
375 possibility is that fragmentation of microbeads occurred during the extraction process. Indeed, for the
376 latter, we observed in a previous experiment that depending on agitation speeds, microbeads could
377 become fragmented due to the mechanical action of the vortexer and/or possible fragility of the beads.

378 Exposure to the smaller RB microbeads had no effect in the colon, but induced some significant
379 modifications in proximal and small intestine. The most notable effect of RB exposure was the decrease
380 of villus/crypt ratio both in proximal and distal small intestine. The villus/crypt ratio was also reduced
381 after exposure to GB alone or with the mixture of both MPs. These ultrastructural changes were
382 primarily due to crypt depth enhancement throughout the small intestine and were not associated with
383 villus atrophy, and therefore reflect hypertrophic crypt formation. The hyperproliferation of the crypt
384 compartment can contribute to intestinal tumorigenesis and therefore deserves further investigation
385 (Murray et al., 2021). RB exposure also downregulated mucin-2 and villin-1 gene expression and
386 decreased recruitment of CD8⁺ T lymphocytes to the proximal small intestine.

387 The larger microbeads, GB, tended to induce more effects in the intestinal tract than RB. Exposure to
388 GB affected 3 parts of the intestine, with a greater impact on colon. At the histological level, we did not
389 observe any significant difference between GB-exposed mice and control mice, showing that it did not
390 induce severe damage in the colon. Significant disturbances were visible by more sensitive methods
391 such as quantitative RT-PCR and flow cytometry. The proportion of neutrophils and the expression of
392 *Vill*, *Chga*, *Lgr5*, *Ocln*, *F11r*, and *Ifng* were enhanced, and the recruitment of anti-inflammatory
393 macrophages was impaired in the colon of GB-exposed mice. These results are in concordance with
394 those of Li et al. who found in similar experimental conditions (10-150 µm PE beads, 200µg/g of food,
395 and 5 week exposure) that TLR4, AP-1, and IRF-5 proteins were upregulated in colon, similarly
396 reflecting a proinflammatory state (B. Li et al., 2020). By contrast, Sun et al. showed that oral exposure
397 to smaller PE microbeads (1 to 10µm) at the dosage of 0.2µg/g bw/d decreased Il1β expression, and
398 increased Il8 and Il10 expression, rather in favor of an immunosuppressive effect of PE of this size (Sun

399 et al., 2021). Therefore, the effects of ingested PE on colonic inflammation appear to be drastically
400 different depending on the size of the microbeads. The difference in effect of RB exposure compared to
401 GB might suggest that even a small difference in particle size at the time of ingestion could influence
402 intestinal toxicity. Consistently, the PS MP uptake into human intestinal epithelial Caco-2 cell line was
403 found greater for 4µm particles than for 1 µm particles (Stock et al., 2019). Furthermore, Sun et al.
404 observed that mouse exposure with PE MPs less than 10 µm in size induced a decreased abundance of
405 Firmicutes and an increased abundance of Bacteroides, whereas these phyla did not vary in our study as
406 well as in the one of Li et al. These findings suggest that the effects of PE MPs on microbiota are also
407 size-dependent, as it has also been observed for PS MPs (Lu et al., 2018)..

408 The metagenomic analysis of microbiota also revealed that PE microbeads of both sizes individually
409 induced same variations of bacteria abundance at the order level. The Verrucomicrobiales were less
410 abundant and the Gastranaerophilales were more abundant in exposed mice. The role of
411 Verrucomicrobiales is not well known but their increase has been associated with the development of
412 acute colitis in the Dextran Sodium sulfate (DSS)-induced model (Jin et al., 2021; R. Li et al., 2020).
413 Gastranaerophilales are more abundant in DSS-induced colitis (Dou et al., 2020), and follow opposite
414 trends in κ-and ι-carrageenan-induced colitis (Shang et al., 2017). These discrepancies are against an
415 essential effect of these bacterial orders in colonic inflammation.

416 Another interesting finding is that the most substantial observed changes occurred following exposure
417 to the mixture of the 2 sizes of PE beads. Firstly, we observed an increase of mucosal and mucin areas
418 and an upregulation of *Muc2*, *Vill*, and *Chga* transcripts in colon reflecting dysregulation of colon
419 mucosa differentiation. An enhancement of *Ocln* and *F11r* expression was also observed suggesting
420 potential barrier dysfunction. The mixture of PE beads induced an increase of *Il6* and *Ifng* expression in
421 favor of a colon proinflammatory state. It also modulated the frequency of CD4⁺ T lymphocytes, CD8⁺
422 T lymphocytes, dendritic cells, and inflammatory monocytes in proximal small intestine, NK cells in
423 distal small intestine, and anti-inflammatory macrophages in colon, showing pronounced alterations of
424 intestinal immune response. Moreover, exposure to the mixture of PE beads decreased the abundance
425 of protective Lactobacillales bacteria (Bartley et al., 2018). Lastly, PE mixture enhanced the frequency

426 of Rhodospirillales, the increased abundance of which has been associated with several pathological
427 conditions such as Damp Heat syndrome (Jiang et al., 2020), neuropsychiatric symptoms in Alzheimer's
428 disease (Zhou et al., 2021), and animal intoxication with N-nitrosamines (Zhu et al., 2019). Therefore,
429 the imbalance observed in the abundance of Rhodospirillales and Lactobacillales could contribute to the
430 negative effects observed following exposure to the 2 types of microbeads, and this deserves to be
431 further explored. Taken together, these results show that, as a mixture, the 2 sizes of PE microbeads
432 more severely affected the homeostasis of intestinal tissues than as single exposure. Liang et al revealed
433 that in mice, co-exposure to a mixture of PS particles of 50 and 500 nm caused more severe dysfunction
434 of the intestinal barrier than that caused by each PS particles individually (Liang et al., 2021). The
435 authors also demonstrated that co-exposure to several sizes of PS particles modulated their
436 biodistribution in mouse organs and increased their bioavailability. The hypothesis can be put forward
437 that, as for PS, the aggravation of the effects that we observed in the group exposed to RB+GB could be
438 partly explained by an increased bioaccumulation of PE microbeads in the event of co-exposure.
439 Interestingly, in the latter study, exposure 50 nm PS particles increased mucus secretion in the
440 duodenum, jejunum and ileum, whereas it was decreased in the colon. The decline of mucus secretion
441 in colon was confirmed following 0.5, 5 and 50 μm PS MP exposure in mice (Jin et al., 2019; Lu et al.,
442 2018). In our work, mucus secretion was respectively decreased in proximal small intestine and
443 increased in colon after co-exposure to 36 and 116 μm PE MPs, whereas smaller sizes PE MPs reduced
444 colon mucin density (Sun et al. 2021). The comparison of the effects observed on this parameter
445 common to several publications shows that the impact of MPs depends on the type of polymer, its size,
446 and the location in the intestine, which underlines the need to continue testing different experimental
447 conditions in order to allow strong advances in the understanding of the MP toxicological effects.

448 Taken together, previous and present studies suggest that PE exposure poses a substantial risk to human
449 intestinal health. Moreover, evidence of a positive correlation between the concentration of fecal MPs
450 and the severity of disease activity (Harvey-Bradshaw index and Mayo score) has been recently reported
451 in a cohort of patients with inflammatory bowel disease (IBD) (Yan et al., 2022). Polyethylene was

452 found in the feces of these patients. Therefore, presence of PE in stool may contribute to the development
453 of inflammation in IBD.

454

455 **Conclusions**

456 The present study demonstrated that a 6-week oral exposure of mice to PE microbeads induced
457 histological, inflammatory, and immune disturbances from the proximal small intestine to the colon.
458 The relative abundance of bacterial orders was also modified. The co-exposure of 2 sizes of PE
459 microbeads led to defects related to gut differentiation, barrier function, and immune response. These
460 alterations of gut response could contribute in the long term to the onset of immune-mediated
461 inflammatory diseases. Human population studies should be performed to correlate PE exposure levels
462 and disease risks.

463 **CRedit authorship contribution statement**

464 MD: Investigation, CV: Conceptualization, Writing-review, Funding acquisition, AD: Investigation,
465 Methodology, Supervision, Writing-original draft, SC: Formal analysis, NH Investigation, CW:
466 Investigation, CH: Investigation, DB: Investigation, DH: Supervision, LD: Funding acquisition, DL:
467 Funding acquisition, GD: Funding acquisition, Supervision, MB-M: Writing-Original draft,
468 Conceptualization, Validation, Formal analysis, Visualization, Supervision, Revision

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475 Declaration of interest

476 No conflict of interest to be declared.

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647 **Figure captions**

648 Fig. 1. Characterization of polyethylene (PE) microbeads. (A-B) Histograms of size distributions and
649 cumulative percentage of red beads (A) and green beads (B) after measurement of 312 particles for each
650 bead category. (C) The combined density plot. (D-E) FTIR profiles obtained for red bead (D) and green
651 bead (E) from 4,000 to 600 cm^{-1} . For each spectrum the red FTIR profile of PE from a custom library is
652 superimposed. (F) Observation of intact (a) and fragmented (b) green beads and intact (c) and
653 fragmented (d) red beads in mouse stool.

654 Fig. 2. Effects of polyethylene (PE) exposure on proximal small intestine epithelium histomorphology
655 and immune response. Mice were exposed to food spiked with 100 $\mu\text{g/g}$ of PE microbeads (red beads
656 (RB), green beads (GB), or RB+GB; $n=10/\text{group}$) or control food (CT; $n=9$) for 6 weeks. (A)
657 Representative pictures of proximal small intestine sections stained with AB-PAS. (B) Villus length and
658 crypt depth. (C) Villus/crypt ratio. (D) Percentage of AB-PAS-positive area. (E) mRNA quantification
659 of markers of intestinal cells *Muc2*, *Vill*, *Chga*, and *Lgr5*. (F) mRNA quantification of tight junction

660 genes *Ocln* and *F11r*. (G) mRNA quantification of inflammatory cytokines *Tnf*, *Ifng*, *Il6*, and *Il1b*. (H)
661 Percentage of significantly changed immune populations: CD4⁺ T lymphocytes, CD8⁺ T lymphocytes,
662 dendritic cells, and inflammatory monocytes. * $p < 0.05$ and ** $p < 0.01$ vs control group as determined
663 by the Mann-Whitney U test.

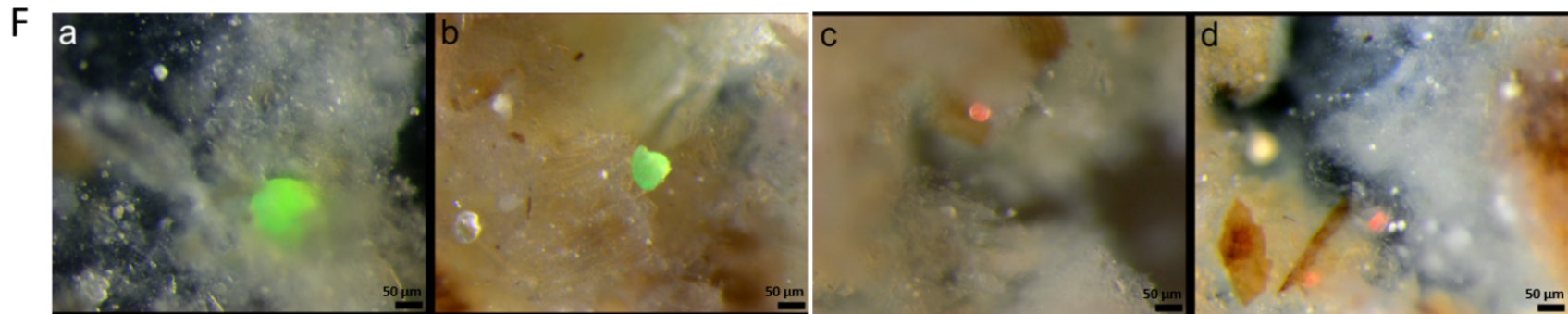
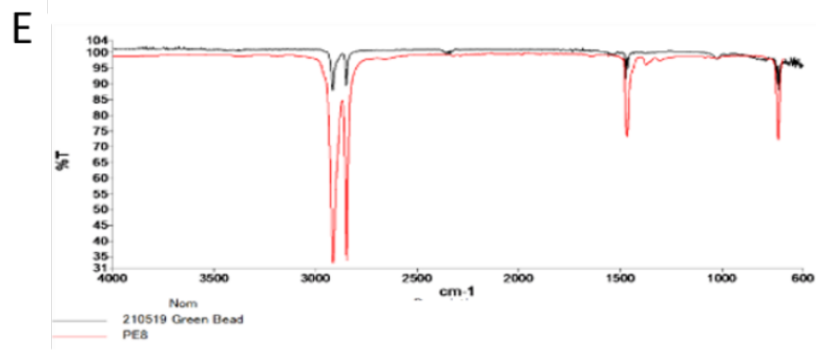
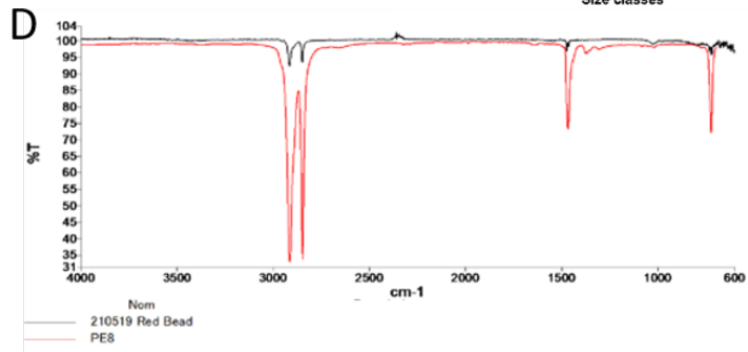
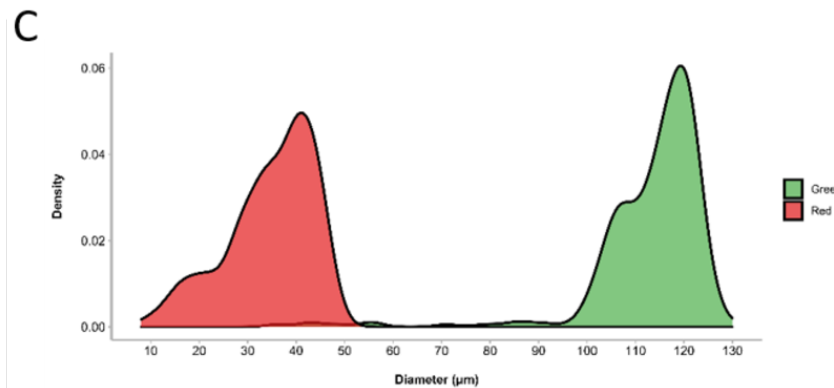
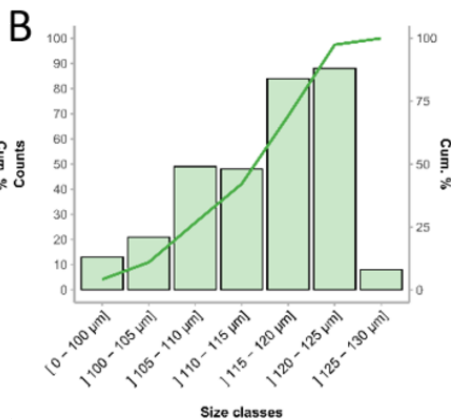
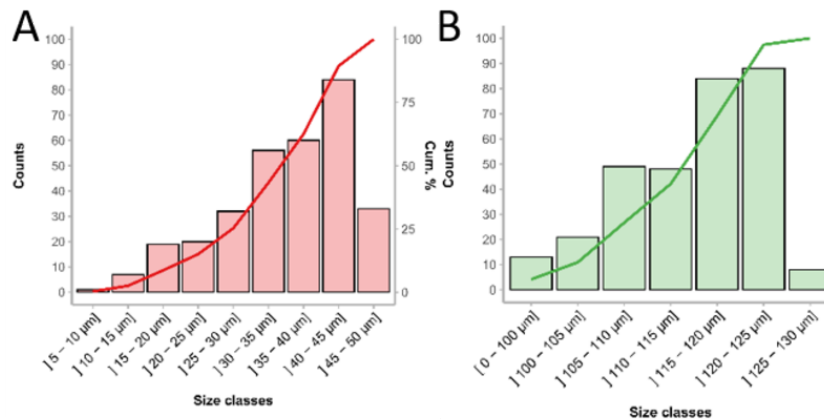
664 Fig. 3. Effects of polyethylene (PE) exposure on distal small intestine epithelium histomorphology and
665 immune response. Mice were exposed to food spiked with 100 $\mu\text{g/g}$ of PE microbeads (red beads (RB),
666 green beads (GB), or RB+GB; n=10/group) or control food (CT; n=9) for 6 weeks. (A) Representative
667 pictures of distal small intestine sections stained with AB-PAS. (B) Villus length and crypt depth. (C)
668 Villus/crypt ratio. (D) Percentage of AB-PAS-positive area. (E) mRNA quantification of markers of
669 intestinal cells *Muc2*, *Vill1*, *Chga*, and *Lgr5*. (F) mRNA quantification of tight junction genes *Ocln* and
670 *F11r*. (G) mRNA quantification of inflammatory cytokines *Tnf*, *Ifng*, *Il6*, and *Il1b*. (H) Percentage of
671 the significantly changed immune population of NK cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, and ****
672 $p < 0.001$ vs control group as determined by the Mann-Whitney U test.

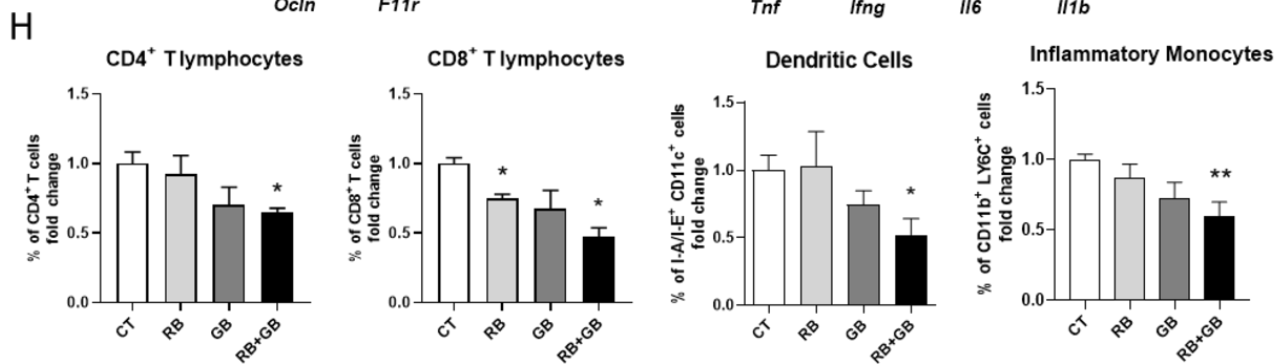
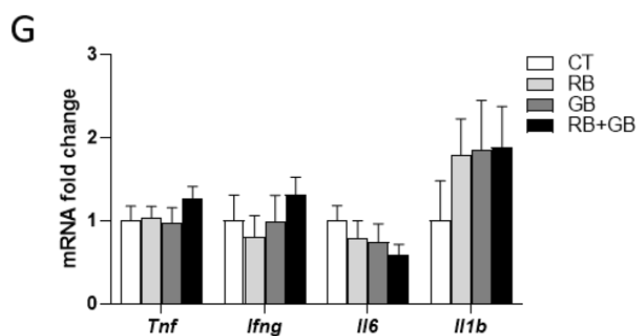
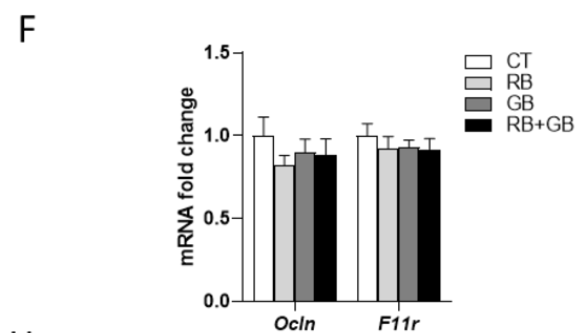
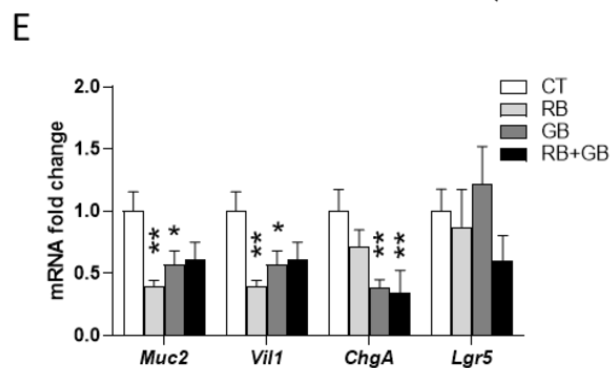
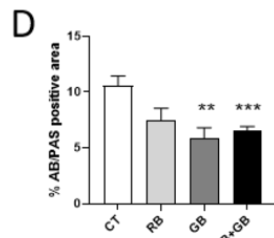
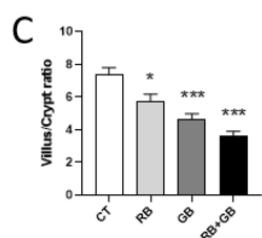
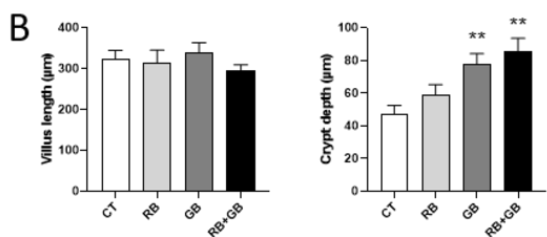
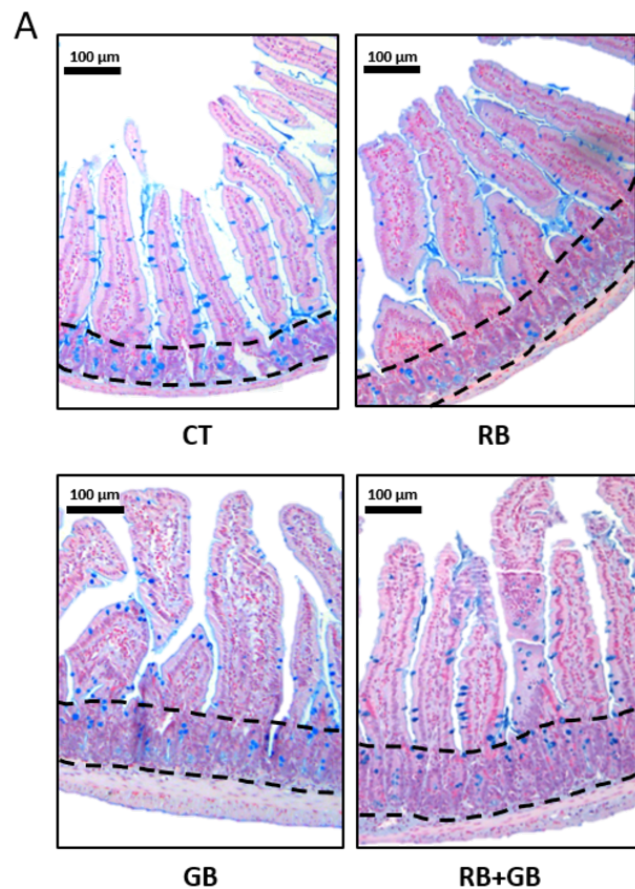
673 Fig. 4. Effects of polyethylene (PE) exposure on colon epithelium histomorphology and immune
674 response. Mice were exposed to food contaminated with 100 $\mu\text{g/g}$ of PE microbeads (red beads (RB),
675 green beads (GB), or RB+GB; n=10/group) or control food (CT; n=9) for 6 weeks. (A) Representative
676 pictures of colon sections stained with AB-PAS. (B) Mucosal surface area. (C) Percentage of AB-PAS-
677 positive area. (D) mRNA quantification of markers of intestinal cells *Muc2*, *Vill1*, *Chga*, and *Lgr5*. (E)
678 mRNA quantification of tight junction genes *Ocln* and *F11r*. (F) mRNA quantification of inflammatory
679 cytokines *Tnf*, *Ifng*, *Il6*, and *Il1b*. (G) Percentage of significantly changed immune populations:
680 polymorphonuclear neutrophils and anti-inflammatory macrophages. * $p < 0.05$, ** $p < 0.01$, and ***
681 $p < 0.005$ vs control group as determined by the Mann-Whitney U test.

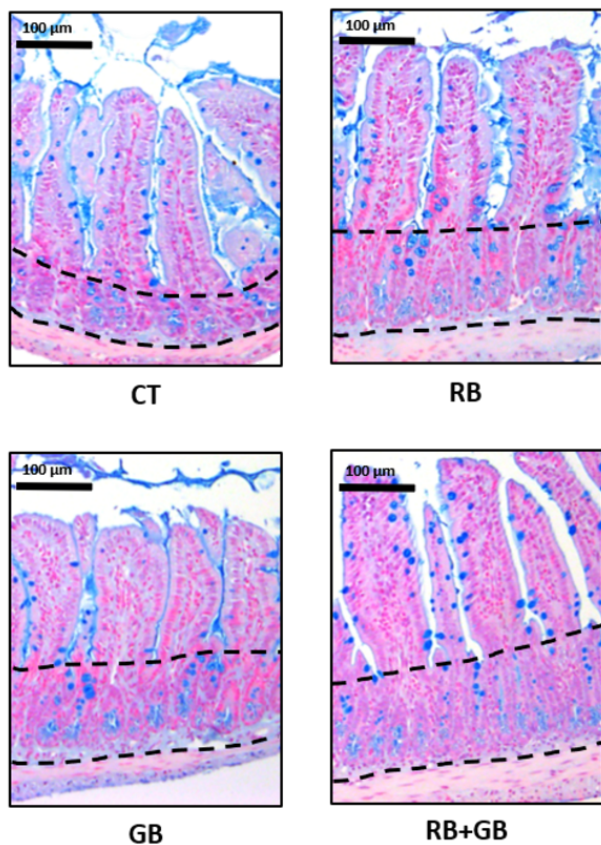
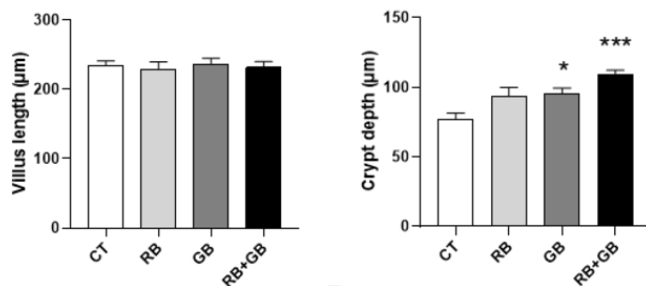
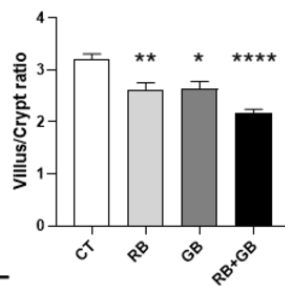
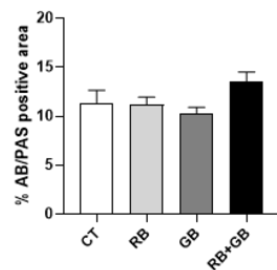
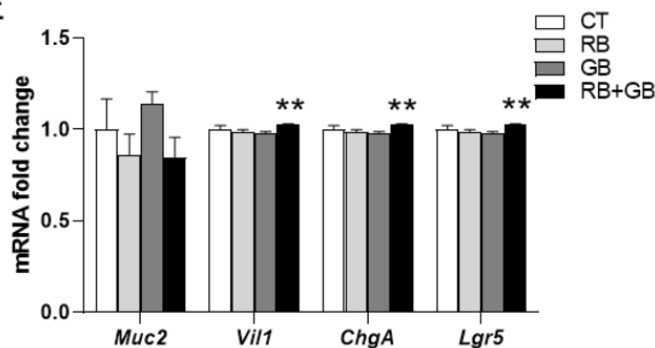
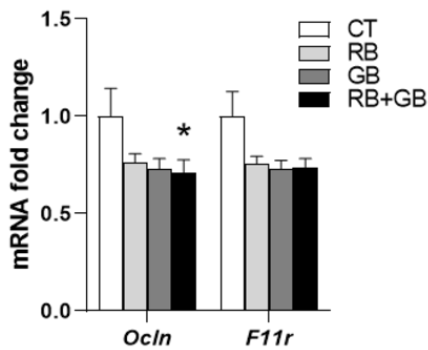
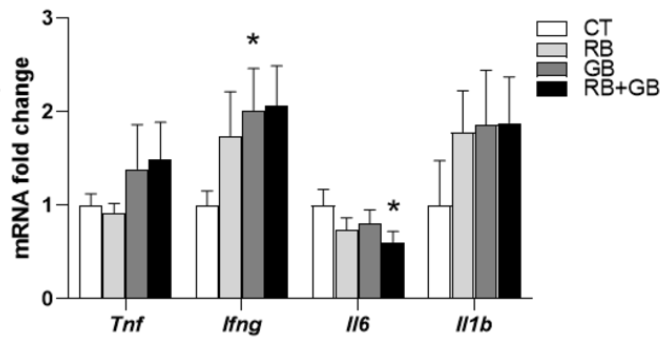
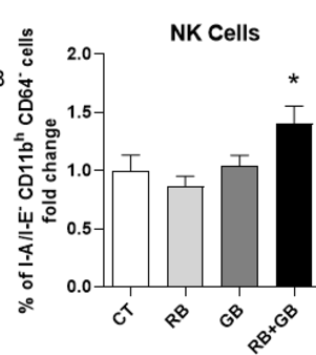
682 Fig. 5. Effects of polyethylene (PE) exposure on the gut microbiome. Mice were exposed to food spiked
683 with 100 $\mu\text{g/g}$ of PE microbeads (red beads (RB), green beads (GB), or RB+GB; n=7-8/group) or control
684 food (CT; n=7) for 6 weeks. (A) Chao1 α diversity index. (B) Unweighted UniFrac β diversity index; *
685 $p < 0.05$ as determined by pairwise PERMANOVA. (C) Overview of the relative abundance of gut

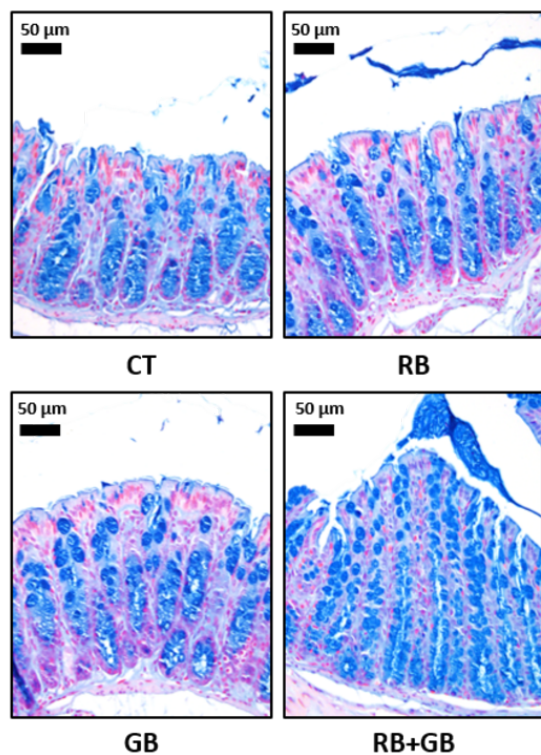
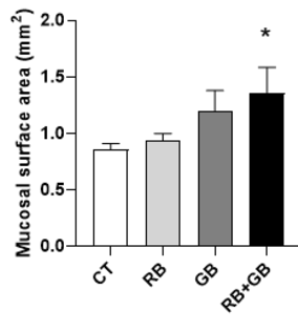
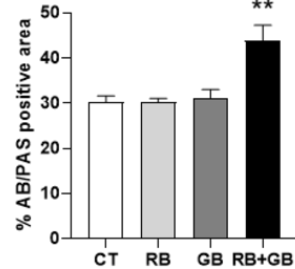
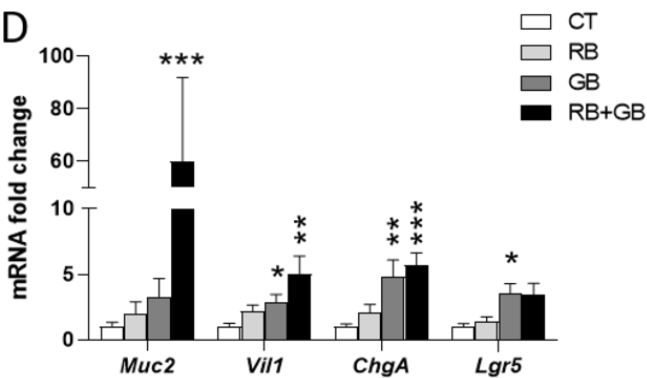
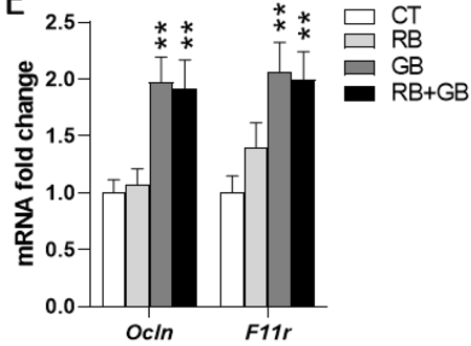
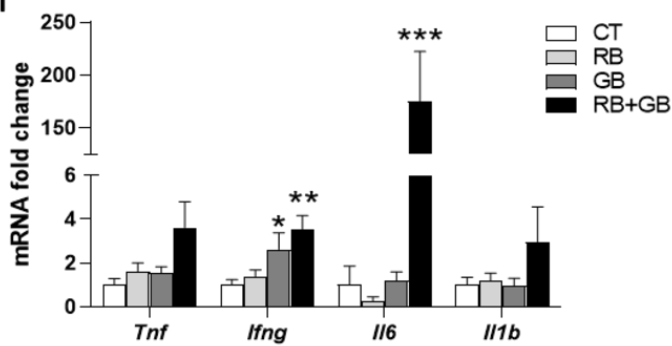
686 bacteria depicted at the phylum level. (D) Differential abundance of significantly changed bacterial
687 orders. * $p < 0.05$ vs control group as determined by Corncob test.

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A**B****C****D****E****F****G****H**

A**B****C****D****E****F****G**