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HAL Id: hal-03791155
https://hal.inrae.fr/hal-03791155

Submitted on 29 Sep 2022

Exposure to polyethylene microplastics alters immature gut microbiome in an infant *in vitro* gut model

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AhR: aryl hydrocarbon receptor; ASV: amplicon sequence variant; DSC: differential scanning calorimetry; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GC-MS: gas chromatography-mass spectrometry; HRP: horseradish peroxydase; HPLC: high-performance liquid chromatography; LDPE: low density polyethylene; LY: lucifer yellow; MP: microplastic; MUC2: mucin 2 ; MUC5AC: mucin 5AC; M-ARCOL: mucosal artificial colon; OCLN: occludin; Papp: apparent permeability coefficient; PE: polyethylene; PET: polyethylene terephthalate; PS: polystyrene; qPCR: quantitative polymerase chain reaction; SCFA: short chain fatty acid; SPME: solid-phase microextraction; TEER: trans-epithelial electrical resistance; TGA: thermogravimetric analysis; TJ: tight junction; Tm-ARCOL: toddler mucosal artificial colon; VOC: volatile organic compound; ZO-1: zonula occludens-1

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24   **Abstract**

25   Infants are characterized by an immaturity of the gut ecosystem and a high exposure to

26   microplastics (MPs) through diet, dust and suckling. However, the bidirectional interactions

27   between MPs and immature infant intestinal microbiota remain unknown. Our study aims to

28   investigate the impact of chronic exposure to polyethylene (PE) MPs on the gut microbiota and

29   intestinal barrier of infants, using the new Toddler mucosal Artificial Colon coupled with a co-

30   culture of epithelial and mucus-secreting cells. Gut microbiota composition was determined by

31   16S metabarcoding and microbial activities were evaluated by gas, short chain fatty acid and

32   volatolomics analyses. Gut barrier integrity was assessed *via* evaluation of intestinal

33   permeability, inflammation and mucus synthesis. Exposure to PE MPs induced gut microbial

34   shifts increasing α-diversity and abundance of potentially harmful pathobionts, such as

35   *Dethiosulfovibrionaceae* and *Enterobacteriaceae*. Those changes were associated to butyrate

36   production decrease and major changes in volatile organic compounds profiles. In contrast, no

37   significant impact of PE MPs on the gut barrier, as mediated by microbial metabolites, was

38   reported. For the first time, this study indicates that ingestion of PE MPs can induce

39   perturbations in the gut microbiome of infants. Next step would be to further investigate the

40   potential vector effect of MPs.

41

42   **Environmental implication**

43   Plastic production has exploded the last half-century, resulting in environmental pollution and

44   microplastics (MPs) contamination throughout the food chain. This raised concerns for human

45   health, particularly in the highly exposed and at-risk infant population. We investigated for the

46   first time the effects of a chronic exposure to polyethylene MPs on immature infant gut

47   microbiome and intestinal barrier, using a novel approach combining a newly developed infant

48   colon model with co-cultures of enterocytes and goblet cells. This work provides useful data

49   on MP interactions with infant microbiota and mucus, considering inter-individualities,

50   increasing our knowledge on the health impact of MP pollution.

51

52   **Keywords**

53   Microplastics, infant, gut microbiota, *in vitro* gut models, cellular models

54

1. Introduction

Plastic is a multifunctional material, easy to process, cheap and resistant. These valuable properties have led to massive plastic production, which began in the 1950's and reaching 367 million tons in 2020 [1]. Various types of plastic are produced around the world, but the most widely manufactured polymer is polyethylene (PE) [1]. Plastic materials are gradually accumulating in all ecological environments, which leads to major environmental threats [2–4]. Subject to weathering and (bio)degradation in the environment, plastic waste releases large amounts of smaller particles that could be sorted by size. Frias and Nash (2019) defined microplastics (MPs) as "any synthetic solid particles or polymeric matrices, with regular or irregular shape and with size ranging from 1 μm to 5 mm, of either primary -voluntarily manufactured- or secondary -released from plastic debris weathering- manufacturing origin, which are insoluble in water" [5].

The ubiquity of MPs throughout the food chain has raised concerns about their potential health effects following ingestion in humans [6–9]. MPs have been detected in air, water and many food and beverage [10], as well as in human blood [11], colon [12] and stools [13–15]. Already in the mother's womb, the fetus seems to be exposed to MPs as they have detected in the placenta and meconium of newborns [16,17]. After birth, infants are highly exposed through breathing and ingesting dust during roaming [18], but also during breastfeeding [19] or bottle feeding, since milk [20,21] and baby bottles [22,23] are contaminated with MPs. In addition, the suckling of silicone rubber represents a source of exposure as steam sterilization leads to the release of MPs [24].

Despite this critical exposure, toxicological studies investigating the potential impact of MPs on infant health remain rare [25,26]. While the embryotoxic effect of polystyrene (PS) nano- or micro-sized plastics seems to vary depending on the model used [27–30], a high dose of PS MPs injected intraperitoneally into pregnant mice resulted in immune disturbance and

80 spontaneous abortion [31]. Oral exposure to PS MPs also induced fecal microbiota dysbiosis

81 and gut barrier dysfunction in dams while offspring showed a higher risk of metabolic disorders

82 [32,33]. Importantly, no studies to date have investigated the bidirectional interactions between

83 ingested MPs and the immature and developing infant microbiome, despite its key role in gut

84 homeostasis. Indeed, during the first 1000 days of life, while the digestive physiology is still

85 under development, the infant intestinal microbiota (which mainly colonizes the colonic

86 compartment) simultaneously undergoes significant maturation in terms of composition and

87 metabolic activity [34–36]. The gut microbiome acts primarily in the degradation of undigested

88 food, resulting in the production of secondary metabolites such as gases, short-chain fatty acids

89 (SCFAs) [37,38], volatile organic compounds (VOCs) [39] and aryl hydrocarbon receptor

90 (AhR) ligands [40]. SCFAs (i.e., butyrate, propionate and acetate) are byproducts of the

91 fermentation of nondigestible dietary fibers under the action of intestinal bacteria, whereas AhR

92 ligands are primarily derived from the metabolism of tryptophan. Both groups of metabolites

93 are involved in maintaining the integrity of intercellular tight junction in the intestinal

94 epithelium, thus linking gut microbial activity to the maintenance of effective intestinal barrier

95 function. In addition, the gut microbiota plays an important role in the formation of host

96 immunity, in the regulation of gut endocrine and neurological functions, and together with the

97 intestinal mucus and epithelium, it forms a protective barrier against exogenous threats such as

98 pathogens and foreign particles [41–43].

99 In this context, as an extension of a previous study by us on adult microbiota [44], we

100 investigated for the first time the impact of oral exposure to PE MPs on the infant microbiome

101 and the intestinal epithelium/mucus barrier. Due to major ethical constraints linked to clinical

102 studies on young populations, but also the striking differences between human and rodent

103 digestive physiology -including microbiota- [45], these experiments were carried out using an

104 innovative *in vitro* approach, combining the Toddler mucosal Artificial Colon (Tm-ARCOL,

105 [46]) simulating the colonic ecosystem specific to infants (6 months to 3 years) and a co-culture

106 of intestinal epithelial and mucus-secreting goblet cells (Caco-2 and HT29-MTX cells,

107 respectively). After a 2-week daily exposure to PE MPs, the structure and metabolic activities

108 of the infant microbiome were determined by 16S metabarcoding and measurements of

109 gas/SCFAs/VOCs/AhR activity, respectively. Fecal water was then applied to intestinal cell co-

110 cultures to assess the impact of gut microbe metabolites on cell-related inflammatory pathways,

111 mucin synthesis and barrier function.

112 **2. Materials and methods**

113 **2.1 Microplastics characterization**

114 PE MPs (CPMS-0.96, 1-10 μm – 0.2 g; non-fluorescent particles) were purchased from

115 Cospheric (CA, USA). The initial powder was suspended (2.625 mg/mL) in a solution of sterile

116 deionized water with 0.01% (w/v) Tween 80 before use. Particle size was determined using the

117 smileview software from scanning electron microscopy images captured with the JSM 6060LV

118 microscope (Jeol, Japan). In order to determine the polymeric composition, the powder was

119 analyzed by differential scanning calorimetry (DSC) and by thermogravimetric analysis (TGA).

120 DSC measurements were performed on a Mettler DSC3$^+$ device (N.V. Mettler-Toledo S.A.,

121 Belgium). The equipment is calibrated with Indium. A few mg of material were placed in an

122 aluminum pan and then subjected to a temperature cycle ranging from 25°C to 200°C under a

123 flow of nitrogen gas. A rate of 10°C/min was applied to heat and cool the material during the

124 experiment. TGA measurements were performed on a Mettler TGA2 device (N.V. Mettler-

125 Toledo S.A., Belgium). The analysis was performed at a heating rate of 10°C/min under a flow

126 of a nitrogen gas up to 800°C.

127 **2.2 Fecal sample collection and treatment**

128 Fecal samples were collected from four healthy infants (two boys aged 30 and 20 months -

129 Toddlers 1 and 3, and two girls aged 25 and 22 months -Toddlers 2 and 4-) were collected. All

130   donors had no history of antibiotic treatment or probiotic consumption during the month prior

131   sample collection. Immediately after defecation, the fecal samples (or diapers) were transferred

132   to a sterile container placed in an airtight anaerobic box (GENbag anaer gas pack systems,

133   Biomerieux, France), transported at ambient temperature and processed in the laboratory within

134   6 h. In an anaerobic chamber (COY laboratories, USA), 11 g of each fresh stool sample were

135   suspended into 110 mL of 30 mM sterile sodium phosphate buffer and filtered (500 μm stainless

136   steel sieve).

137   **2.3   Description and set-up of the colonic Tm-ARCOL model**

138   The Tm-ARCOL is a one-stage fermentation system (MiniBio, Applikon, The Netherlands),

139   operated under continuous conditions, which simulates both physicochemical (pH, retention

140   time, ileal effluents supply and anaerobiosis) and microbial conditions (lumen and mucus

141   associated microbiota) encountered in the colon of young infant [46]. This model is composed

142   of a main bioreactor (luminal compartment) connected to an external glass compartment

143   (mucosal compartment) containing mucin-alginate beads (**Fig. 1a**). These beads were prepared

144   with type II porcine stomach mucin (Sigma-Aldrich, USA) and sodium alginate (Sigma-

145   Aldrich, USA), as previously described [47]. The mucin-alginate beads were stored at 4°C

146   before introduction into the hermetically sealed glass compartment (total beads area of 556 cm²)

147   connected to the bioreactor. Tm-ARCOL was set-up, based on *in vivo* data, to reproduce the

148   average conditions found in the colon of a healthy toddler, as previously described [46]. Briefly,

149   the composition of the ileal effluents was adjusted to reflect the specificities of the infant diet

150   (**Fig. 1a**). The temperature of the main bioreactor and mucin-alginate bead compartment was

151   maintained at 37°C, the average retention time was set at 19 h and the pH was maintained at a

152   constant value of 6.1 by addition of 2M NaOH. After an initial N_2 flush upon introduction of

153   the fecal inoculum, anaerobiosis was maintained inside the bioreactor by the activity of the

154   resident microbiota alone, in order to faithfully reproduce the *in vivo* situation. The mucin-

155 alginate beads were renewed every two days while maintaining the external compartment in

156 anaerobiosis thanks to a constant flow of CO_2.

2.4   Experimental design and sampling during *in vitro* fermentations

158 The experimental design and sampling are summarized in **Fig. 1b**. Four Tm-ARCOL

159 bioreactors were inoculated with fecal samples from the four infant donors and operated in

160 parallel. Fermentations were carried out under continuous conditions for 22 days, after a 24-h

161 batch microbial amplification. An eight-day microbiota stabilization phase was applied and the

162 three last days of this period (days 6, 7 and 8) were chosen as control. Then, fourteen days of

163 daily exposure to PE MPs (21 mg of MPs in 8 mL of a solution of deionized water containing

164 0.01% (w/v) Tween 80) were carried out and the three last days (days 20, 21 and 22) were

165 chosen as exposure days. Samples from the main bioreactors, termed luminal microbiota, were

166 collected daily for microbiome characterization (storage at -80°C) and SCFA analysis (storage

167 at -20°C). Additional samples from the mucin-alginate bead compartment, termed mucosal

168 microbiota, were collected every other day (when mucin-alginate beads were replaced) for

169 microbiota characterization. Beads were washed twice in sterile phosphate buffer saline -PBS-

170 (pH 7.1) and stored at -80°C before downstream analyses. The medium surrounding mucin-

171 alginate beads was also collected at the same times in case of insufficient amounts of DNA on

172 the mucin-alginate beads. Additional luminal fermentation media were collected on day 8

173 (control) and day 22 (exposure) for cell culture experiments, AhR activity measurement,

174 scanning electron microscopy, Raman spectroscopy and volatolomic analysis. Samples were

175 also collected daily from the atmospheric phase of the bioreactors to verify anaerobic conditions

176 and determine gas composition. The daily extra volume of gas produced by microbial

177 fermentation was also measured using a syringe connected to the gas bag.

2.5 DNA extraction

178 **2.5** **DNA extraction**

179 Genomic DNA was extracted from luminal samples and mucin-alginate beads (or surrounding

180 medium) using the QIAamp Fast DNA Stool Mini Kit (12830-50, Qiagen, Germany) following

181 the manufacturer's instructions with minor adjustments. Prior to DNA extraction, luminal

182 samples were centrifuged (2000 g, 10 min, 4°C) and the pellet was mechanically disrupted

183 using a bead beater (5 min, 20 beat/sec) with 300 mg of sterile glass beads (diameter ranging

184 from 0.1 to 0.6 mm), incubated (70°C, 5 min) and centrifuged (12000 g, 1 min, 4°C). Mucin-

185 alginate beads were subjected to the following modifications prior to DNA extraction: 10 min

186 of incubation with citrate buffer (37°C), as previously described [48], before vortexing

187 (maximal speed, 3 min) and centrifugation (8000 g, 1 min). DNA integrity was verified by

188 agarose gel electrophoresis and Nanodrop 2000 analysis (Thermo Fisher Scientific, USA).

189 DNA quantity was assessed using the Qubit dsDNA Broad Range Assay Kit (Q32851,

190 Invitrogen, USA) with a Qubit 2.0 Fluorometer (Invitrogen, USA). Samples were stored at -

191 20°C before gut microbiota analysis (qPCR and 16S metabarcoding).

192 **2.6** **Total bacteria quantification by qPCR**

193 Total bacteria were quantified by qPCR using primers BAC338R and BAC516F with an

194 hybridization temperature set at 58°C [49] (**Table 1**). Real-time PCR assays were performed

195 on a Biorad CFX96TM Real-Time System (Bio-Rad Laboratories, USA) using the Takyon™

196 Low Rox SYBR® 2X MasterMix blue dTTP kit (B0701, Eurogentec, Belgium). Each reaction

197 was performed in duplicate in a final volume of 10 μL with 5 μL of Master Mix, 0.45 μL of

198 each primer (10 μM), 1 μL of DNA sample (10 ng/μL) and 3.1 μL of ultra-pure water. The

199 amplifications were performed as follows: 1 cycle at 95°C for 5 min, followed by 40 cycles at

200 95°C for 30 s and 60°C for 1 min. A melting step was added to ensure the specificity of the

201 primers. The standard curve was generated from 10-fold dilutions of bacterial DNA as

202 described in [47].

203 **2.7   16S metabarcoding and data analysis**

204 The bacterial V3-V4 region of the 16S ribosomal DNA (rDNA) was amplified using primers

205 V3_F357_N and V4_R805 for bacterial fraction and Arch349F and Arch806R for

206 methanogenic Archaea. Amplicons were generated using a Fluidigm Access Array followed by

207 high-throughput sequencing on an Illumina MiSeq system performed at the Carver

208 Biotechnology Center of the University of Illinois (Urbana, USA).

209 Bioinformatics analysis was performed by GeT-Biopuces platform (INSA/Toulouse

210 Biotechnology Institute, Toulouse, France) with R software version 4.1.1 (2021-08-10) and

211 rANOMALY package [50]. The demultiplexed raw sequence data were filtered by quality and

212 the denoising process performed using DADA2 version 1.20.0 [51]. Reads with N bases or low

213 phred quality score (under 2) were eliminated and reads under 100 pb length removed.

214 Decontamination steps were carried out to filter out sequences corresponding to PhiX DNA

215 used as a spike-in control for MiSeq runs and chimeric sequences were filtered out. Taxonomic

216 affiliation of all amplicon sequence variants (ASVs) was performed with idtaxa function from

217 DECIPHER package version 2.20.0 [52] using SILVA release 138 [53] and GTDB

218 bac120_arc122 [54] databases (60% bootstrap cut-off). To improve completeness of taxonomic

219 affiliation, alignments were carried out using BLAST [55] (98% identity and coverage) on

220 representative sequences of unassigned ASVs or assigned with incomplete taxonomy. A

221 phylogenetic tree was constructed based on the representative ASVs sequences using functions

222 from phangorn package version 2.7.1 [56]. α- and β-diversity indexes were calculated using the

223 diversity_alpha_fun function from rANOMALY package [50]. The across-sample microbiota

224 diversity was described using redundancy analysis (RDA) based on Bray-Curtis dissimilarities

225 using vegan R-package version 2.5-7 [57]. The impact of the different parameters analyzed

226 (total gas and SCFA production, gas and SCFA composition, luminal or mucosal sample types,

227 time and sex of donors) on dissimilarities among groups was evaluated using PERMANOVA

228 permutation tests (999 permutations).

2.8 Gas analysis

230 The analysis of O_2, N_2, CO_2, CH_4 and H_2 gases produced during the fermentation process in the

231 atmospheric phase of the main bioreactor was performed using a HP 6890 gas chromatograph

232 (Agilent Technologies, USA) coupled to a micro-TCD detector (Agilent Technologies, USA).

233 Two series of columns, Molecular Sieve 5A and Porapack Q (Agilent Technologies, USA) were

234 used. The composition of the gases was determined using calibration curves produced from

235 ambient air (78.09 % N_2, 20.95 % O_2, 0.04 % CO_2) and 3 gas mixtures A (5 % CO_2, 5 % H_2,

236 90 % N_2), B (19.98 % CO_2, 80.02 % H_2) and C (19.89 % CO_2, 19.88 % CH_4, 20 % H_2, 40.23

237 % N_2).

2.9 Analysis of short chain fatty acid composition

239 2 mL of each luminal sample was centrifuged (5000 g, 15 min, 4°C) and 900 µL of the

240 supernatant was diluted at 1/10 in 0.04 M H_2SO_4 mobile phase, vortexed and filtered (pore size

241 0.22 µm). The three major SCFAs (acetate, propionate and butyrate) were quantified by high-

242 performance liquid chromatography (HPLC) (Elite LaChrom, Merck HITACHI, USA) coupled

243 to a DAD diode. The HPLC column (150 x 7.8 mm) contained a negatively charged sulfonic-

244 grafted polystyrene divinylbenzene stationary phase and carried an eluent containing acidified

245 water. Data were obtained and analyzed by the EZChrom Elite software at 204 and 205 nm.

246 SCFAconcentrations were calculated from calibration curves established from solutions of

247 known increasing concentration of acetate, propionate and butyrate (0, 10, 25 and 40 mM).

2.10 Measurement of AhR activity

249 The AhR activity of Tm-ARCOL luminal supernatants after exposure to PE MPs (day 22)

250 relative to control conditions (day 8) was measured using a luciferase reporter assay method,

251 as previously described [58]. H1L1.1c2 mouse hepatocellular carcinoma cells, containing a

252   stably integrated dioxin response element-driven firefly luciferase reporter plasmid

253   pGudLuc1.1, were seeded in 96-well plates at 10^5 cells/well in Minimum Essential Medium

254   Eagle Alpha Modification (MEM-α) medium (with 10% (v/v) heat-inactivated fetal calf serum

255   -FCS, 1% (v/v) penicillin/streptomycin, 1% (v/v) Geneticin G418) and cultured (37°C, 5%

256   CO_2) 24 h before stimulation with the Tm-ARCOL supernatants for 24 h. After incubation, the

257   wells were washed with 100 μL PBS (pH 7), and 50 μL of Promega lysis buffer (pH 7.8) was

258   added to each well. The plates were shaken for 1 h for cell lysis. After addition of 100 μL of

259   luciferase reagent (Promega, France), the luciferase activity was measured using a luminometer

260   (Tecan, Switzerland). The experiments were performed in triplicate. All values were

261   normalized for sample cytotoxicity using the Lactate Dehydrogenase Activity Assay (Promega,

262   USA).

263   **2.11   Volatolomic analysis**

264   Volatile organic compounds (VOCs) from control (day 8) and exposed (day 22) luminal

265   samples were analyzed by solid-phase microextraction (SPME) coupled with gas

266   chromatography-mass spectrometry (GC-MS), as previously described [59,60]. Briefly, the day

267   before analysis, 2.2 mL of saturated NaCl solution (360 g/L) was added to each sample (0.8

268   mL). The mixing vials were closed under a N_2 flow, vortexed for homogenization and thawed

269   for 24 h at 4°C. The following steps were then performed with an automated sampler (AOC-

270   5000 Shimadzu, Japan): (i) preheating of the sample to 40°C for 10 min in the shaker (500 rpm),

271   (ii) SPME trapping (75 μm carboxen/polydimethylsiloxane, 23-gauge needle, Supelco, USA)

272   of the VOCs for 30 min at 40°C, and (iii) thermal desorption at 250°C for 2 min in splitless

273   mode in the GC inlet. Further analysis of VOCs was performed by GC/MS-full scan (GC2010,

274   QP2010+, Shimadzu, Japan). VOCs were injected in a DB-5MS capillary column (60 m × 0.32

275   mm × 1 μm, Agilent, USA) according to the GC-MS parameters used by Defois and

276   collaborators [59,60]. Provisional identification of VOCs was performed on the basis of mass

277 spectra, by comparison with mass spectra libraries (Wiley Registry 12th Edition / NIST 2020),

278 and of retention indices (RI), by comparison with published RI values and with those of our in-

279 house database. The peak area of the tentatively identified compounds was determined for each

280 of the targeted molecules using a mass fragment selected for its specificity and lack from co-

281 elution.

282 **2.12   Scanning electron microscopy analysis**

283 The luminal and mucosal samples of the Toddlers 2 and 3, one girl and one boy, were deposited

284 on SEMPore filters (Jeol, Japan) and fixed for 12 h at 4°C in 0.2 M sodium cacodylate buffer

285 at pH 7.4 containing 4% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde. The filters

286 were washed for 10 min in sodium cacodylate buffer (0.2 M, pH 7.4) and post-fixed for 1 h

287 with 1% (w/v) osmium tetroxide in the same buffer. The filters were then washed 20 min in

288 distilled water. Gradual ethanol dehydration was performed from 25° to 100° (10 min each) and

289 completed in hexamethyldisilazane (HMDS) for 10 min. The samples were mounted on stubs

290 using adhesive carbon tabs and sputter-coated with gold-palladium (JFC-1300, Jeol, Japan).

291 The analysis was carried out using a scanning electron microscope JSM-6060LV (Jeol, Japan)

292 at 5kV in high vacuum mode.

293 **2.13   Raman spectroscopy mapping**

294 Mapping by Raman spectroscopy was carried out using a Raman spectrometer (Alpha300R

295 Apyron, Witec, Germany) equipped with a confocal microscope. Exposed luminal samples (day

296 22, Toddler 3) were vacuum filtered using an Anodisc filter (pore size 0.2 μm, diameter 25

297 mm). The filters were excited with a Coherent sapphire laser (532 nm, laser power: 10 mW)

298 and Raman spectra were recorded in backscattering configuration using a 50X objective (Zeiss,

299 Germany) with a numerical aperture of 0.55 to achieve a theoretical spatial resolution of 590

300 nm in the focal plane. Individual Raman spectra were recorded 10 times with an integration

301 time of 1 s. For each filter, maps between 900 and 2500 spectra were recorded with a step of

302   1.33 µm and 2 µm, respectively. For each mapping, the recorded signal (Raman signal plus

303   background) was integrated over a 20 cm^{-1} spectral range of centered at 1294 cm^{-1}

304   corresponding to the CH_2 twisting vibration band of PE. The signal value was then

305   automatically transformed by the software in a color scale (from black to bright yellow).

2.14   Intestinal cell lines and cell culture conditions

307   The human colon adenocarcinoma Caco-2 cell line was obtained from the European Collection

308   of Cell Cultures (ECACC, UK) and used at passage 58-62. The mucus-secreting colon

309   adenocarcinoma HT29-MTX cell line was kindly provided by Dr. Thécla Lesuffleur (INSERM

310   Lille, France) and used at passage 10-16. Both cell lines were maintained in cell culture medium

311   (Dulbecco/Vogt modified Eagle's minimal essential medium -DMEM- without phenol red and

312   4.5 g/L glucose), supplemented with 1% (v/v) penicillin/streptomycin, 1% (v/v) Non-Essential

313   Amino Acids –NEAA-, 1% (v/v) Glutamax, and 10% (v/v) heat-inactivated fetal bovine serum

314   -FBS- at 37°C in a humidified atmosphere of 5% CO_2/95% air. The medium was changed every

315   two to three days and cells were split at 80% of confluence using Tryple express. The intestinal

316   cells (90% Caco-2/10% HT29-MTX) were seeded in 24-well inserts with 1-µm pore size

317   (Millipore, Merck Millipore SAS, France) and maintained in the culture medium. From day 17,

318   FBS of the culture medium was replaced by 1% (v/v) Insulin Transferrin Selenium (ITS) in

319   order to limit any interference with the samples to be tested. After 18 days, Caco-2/HT29-MTX

320   co-cultures were incubated apically for 24 h with Tm-ARCOL luminal samples taken on day 8

321   (control) and day 22 (exposed), centrifuged (5000 g, 15 min, 4°C) and diluted 20-foldin cell

322   culture medium (**Fig. 1c**). Experiments were performed with 2 biological replicates and 5

323   technical replicates for each donor.

2.15   Cell viability

325   In order to determine the potential cytotoxic effect of Tm-ARCOL luminal supernatants on

326   Caco-2/HT29-MTX cells and to establish subtoxic conditions for testing on inserts, different

327 dilutions (1/2, 1/5, 1/10, 1/20, 1/30 and 1/50 in cell culture medium) were evaluated. Cell

328 viability was measured in triplicate with CytoTox 96® non-radioactive cytotoxicity test

329 (Promega, France) after 24 h of exposure. In 96-well plates, 25 µL of apical cell culture

330 supernatants were diluted in 25 µL of 1% (v/v) ITS culture medium and mixed with 50 µL of

331 substrate. The plates were then incubated for 15 min at room temperature in the dark. The

332 reaction was stopped with 50 µL of stop solution (1M CH_3COOH) and absorbance at 490 nm

333 was measured with a Spark spectrophotometer (Tecan, Switzerland).

334 **2.16 Intestinal permeability**

335 Starting on day 12, trans-epithelial electrical resistance (TEER) was continuously monitored in

336 real time using the CellZScope2 device (NanoAnalytics, Germany). In addition, after a 24-h

337 contact time with the 20-fold diluted luminal supernatants of Tm-ARCOL, the inserts were

338 transferred to a new 24-well plate containing 1 mL of Hank's balanced salt solution -HBSS.

339 Lucifer yellow -LY- (0.4 mg/mL, molecular weight 457 g/mol) and horseradish peroxydase -

340 HRP- (0.4 mg/mL, molecular weight 44,000 g/mol) were dissolved in HBSS and added to the

341 apical compartment. After 2 h at 37°C under an atmosphere of 5% CO_2/95% air, samples were

342 taken from the apical and basolateral compartments. For LY measurement, samples were

343 analyzed in duplicate using a Spark multimode plate reader (Tecan, Switzerland). Excitation

344 and emission wavelengths were 405 nm and 535 nm, respectively. Total HRP was determined

345 by an ELISA assay. Briefly, 96-well black flat-bottom plates (Greiner, Dutcher, France) were

346 coated overnight at 4°C with 50 µL of 10 µg/mL HRP mouse polyclonal (Abcam, France) in

347 PBS. Plates were blocked with PBS-1% bovine serum albumin (BSA) before incubation with

348 apical and basolateral samples collected from inserts. Rabbit polyclonal anti-HRP biotin

349 (Abcam, France) was added at a concentration of 10 µg/mL before adding Fluorescein-5-

350 isothiocyanate -FITC- conjugated streptavidin (Becton Dickinson, France) for 20 min and

351 fluorescence intensity was measured at 485 nm/525 nm using a Spark microplate reader (Tecan,

352 Switzerland). The apparent permeability coefficient (Papp in cm/s) was determined by the

353 following equation: Papp=Cb/(SxC0), where Cb represents the level of accumulation of LY or

354 HRP in the basolateral compartment after 2 h, S represents the surface of the membrane (0.33

355 cm²) and C0 is the initial concentration of the marker (0.4 mg/mL -LY- or -HRP-) in the apical

356 compartment.

357 **2.17   Interleukin-8 level measurement**

358 The apical and basolateral media of the co-cultures were collected after the contact time of 24

359 h with the 20-fold diluted luminal supernatants of Tm-ARCOL and stored at -20°C until

360 analysis. Interleulin-8 (IL-8) levels were measured using the Quantikine Human IL-8 ELISA

361 kit (DY208-05, R&D Systems, USA) following the manufacturer's instructions. Briefly, in

362 Maxisorp 96-well plates (Nunc, France), biotinylated Goat polyclonal anti IL-8 was added at a

363 concentration of 10 ng/mL before adding HRP-conjugated streptavidin (Becton Dickinson,

364 France) for 20 min. Absorbance intensity was measured at 450 nm and 540 nm using a Spark

365 microplate reader (Tecan, Switzerland).

366 **2.18   Intestinal cell gene expression**

367 Total RNAs were extracted from cells for each insert using the AllPrep RNA Mini kit (Qiagen,

368 France) according to the manufacturer's instructions. Quantity and quality of extracted RNA

369 samples were evaluated using Nanodrop (Nanophotometer Implen-Dutscher, France) and

370 Bioanalyzer (Agilent Technologies, USA), respectively. RNAs were reverse transcribed using

371 enzyme IScript reverse transcription supermix (Biorad, France). Sample concentration was

372 adjusted to 50 ng/µL. 384-well plates were filled by an Agilent Bravo Automated Liquid

373 Handling Platform (Agilent Technologies, France). All wells contained 5 µL of the following

374 mix: 2.5 µL of IQ SYBR green Supermix (Biorad, France), 1.5 µL of each primer set and 1 µL

375 of cDNA sample. Amplification was performed using a ViiA7 Real-Time PCR System

376 (Applied Biosystems-ThermoFisher Scientific, France). Thermal cycling conditions were as

377 follows: 3 min denaturation at 95°C followed by 40 cycles at 95°C for 15 s and 45 s at 60°C,

378 and a melting curve step. The primer sequences of the targeted genes (genes encoding tight

379 junction (TJ) proteins (*ZO-1*, *OCLN*) and genes involved in mucus synthesis (*MUC2*,

380 *MUC5AC*) are listed in **Table 1**. Raw data that passed quality control were analyzed with

381 LinRegPCR (version 2021.2) and then normalized against the expression of glyceraldehyde-3-

382 phosphate dehydrogenase (*GAPDH*), a reference housekeeping gene.

383 **2.19   Statistical analysis**

384 Statistical analyses on gut microbiota activity (gas, SCFAs, AhR activity), α-diversity indexes

385 (number of observed ASVs and Shannon index) from metabarcoding data, as well as results

386 from cell culture experiments, obtained in control and exposed groups, were performed using

387 GraphPad Prism software version 9.3.1 for Windows (GraphPad Software, USA). Data normal

388 distribution was verified by combining Anderson-Darling, D'Agostino & Pearson, Shapiro-

389 Wilk and Kolmogorov-Smirnov tests and homoscedasticity was checked using the Fisher test.

390 Then, appropriate statistical analysis was applied (either one-way ANOVA, Kruskal-Wallis or

391 Welch's tests) and significance was considered for $p<0.05$. For gut microbiota results,

392 differential analyses (DESeq2, metagenomeSeq, metacoder) were performed on

393 ExploreMetabar software using rANOMALY package [50,61]. Principal coordinate analysis

394 (PCoA) of unweight UniFrac distances was performed and significance between groups was

395 assessed with a permutational multivariate analysis of variance (PERMANOVA) using

396 ADONIS (999 permutations) with non-parametric tests. Volatolomics data were processed

397 using the Statistica Software (v.13) (StatSoft, France). Student's t-tests ($p<0.05$) were carried

398 out for the VOC abundances determined in control (day 8) and exposed (day 22) groups and

399 principal component analyses (PCA) were performed on the discriminant VOCs selected to

400 visualize the structure of the data.

3. Results

3.1 Characteristics of PE MPs and interactions with the infant gut microbiota

DSC and TGA analysis of PE MPs showed the purity of the PE powder and characterized it as low-density PE. PE MP microscopy count indicated a size between 1 and 2 μm for 50% of 156 beads analysed [44]. Scanning electron microscopy of samples collected in the luminal phase and mucosal beads in Tm-ARCOL (**Fig. 2a**) showed colonization of the surface of PE MPs by various bacteria. In addition, Raman spectroscopy mapping of PE MPs in the luminal medium (**Fig. 2b**) after exposure provided the same spectrum as that obtained for the initial PE particles, indicating the absence of physicochemical changes to the MP surface during fermentation in Tm-ARCOL.

3.2 Impact of exposure to PE MPs on the infant gut microbiota composition

The stool microbiota of toddlers used for Tm-ARCOL inoculation was characterized by 16S metabarcoding (**Fig.S1**) and showed an absence of methanogenic Archaea and bacterial profiles highly donor-dependent, particularly at the family level. Notably, the stool of Toddler 1 was characterized by a high abundance of *Tannerellaceae*, while *Enterobacteriaceae* and *Akkermansiaceae* were found mainly in Toddler 2 and *Sutterellacaea* and *Oscillospiraceae* in Toddler 4. α-diversity was also donor-dependent (data not shown), with the highest values for Toddlers 1 and 4 (107 and 145 ASVs, respectively) and the lowest for Toddlers 2 and 3 (54 and 70, respectively). Some of these stool individual specificities was captured in Tm-ARCOL, such as *Tannerellaceae* in Toddler 1 and *Enterobacteriaceae* in Toddler 2 (**Fig. 3a**) and a clearly higher number of ASVs under control conditions of Toddler 4 relative to other toddlers (**Fig. 4a**). At the end of the stabilization phase in Tm-ARCOL (control), the bacterial abundance profiles at the family (**Fig. 3a**) and phylum (**Fig. S2a**) levels clearly indicated that the inter-individual variability observed in the initial donor stools was retained in the *in vitro* model, with the donor effect being the most important factor explaining luminal and mucosal β-diversity

426 (**Fig. S2b**). These data also highlighted clear differences between lumen and mucus-associated

427 microbiota and, as expected, their stability at the end of the stabilization phase. After 14 days

428 of daily exposure to PE MPs, clear shifts in bacterial populations were observed both in the

429 luminal and mucosal compartments, and both at the phylum (**Fig. S2a**) and family (**Fig. 3a**)

430 levels. Those changes were again donor-dependent. For instance, exposure to PE MPs resulted

431 in disappearance of *Verrucomicrobia* (*Akkermansiaceae*) in Toddler 2 whereas opposite trends

432 were seen in Toddler 4 and *Synergistetes* (*Dethiosulfovibrionaceae*) increased only in Toddler

433 3. Exposure to PE MPs also induced in the luminal phase a loss in *Ruminococcaceae* in Toddlers

434 1 and 2 while *Rikenellaceae* and *Sutterellaceae* decreased in Toddlers 2 and 4. In addition, the

435 population of *Tannerellaceae* population, which was only present in Toddler 1, disappeared

436 from the mucosal phase after exposure to MPs whereas *Enterobacteriaceae* increased in mucus-

437 associated bacteria from Toddlers 2 and 4. Differential analysis of gut microbiota composition

438 in all donors confirmed significant changes in bacterial populations. In the luminal phase,

439 abundance of *Dethiosulfovibrionaceae*, *Planococcaceae*, *Enterobacteriaceae*, *Moraxellaceae*,

440 *Acidaminococcaceae* and *Oscillospiraceae* increased significantly ($p<0.05$, n=4) after exposure

441 to PE MPs, while the proportion of *Tannerellaceae*, *Rikenellaceae* and *Monoglobaceae*

442 decreased (**Fig. 3b**).

443 Fewer populations were affected in the mucosal compartment, with an increased abundance of

444 *Dethiosulfovibrionaceae*, *Planococcaceae*, *Acidaminococcaceae* and *Oscillospiraceae* but a

445 loss in *Lachnospiraceae* and *Monoglobaceae* after exposure to PE MPs. The impact of such

446 exposure on microbiota structure was confirmed by β-diversity analysis, which showed

447 clustering between control and exposed samples (**Fig. 4c**), with a greater impact in Toddlers 1,

448 2 and 3 compared to Toddler 4. Accordingly, MP treatment also resulted in a significant

449 increase in observed ASVs ($p<0.01$) in the luminal microbiota only for Toddlers 1, 2 and 3.

450 Similarly, in mucosal samples, the number of observed ASVs tended to increase in Toddlers 1

451 and 2, and significance was reached in Toddler 3 ($p<0.05$). Finally, RDA analysis of bacterial

452 β-diversity, based on both 16S metabarcoding and metabolic activity data (see 3.3), indicated

453 that exposure to PE MPs remained the most important parameter explaining variabilities.

3.3 Impact of exposure to PE MPs on the metabolic activities of human infant microbiota

456 In parallel to microbiota composition, the impact of exposure to PE MPs was also monitored

457 *via* gut microbial activities, first by monitoring fermentation gas and SCFAs daily (**Fig. S3**).

458 Similarly to the microbiota composition described above, this analysis showed stable gas and

459 SCFA profiles for each donor at the end of the stabilization period, with the exception of

460 Toddler 3 due to technical problems. Exposure to PE MPs had very limited impact on total gas

461 and SCFA production (**Fig. 5a** and **5b**). Regarding the production of major SCFAs (**Fig. 5c**),

462 acetate tended to increase after exposure to PE MPs for Toddler 1, 2 and 3, while the opposite

463 trend was observed for propionate. Butyrate production dropped after exposure to PE MPs for

464 all donors with significance reached only for Toddlers 1 ($p<0.0001$) and 4 ($p<0.05$), and when

465 data were averaged ($p<0.0001$, n=4). The ability of the gut microbiota to produce ligands

466 capable to activate AhR was also determined in Tm-ARCOL supernatants after exposure to PE

467 MPs (day 22) compared to control conditions (day 8) (**Fig. 6**). After exposure to PE MPs,

468 luminal contents tended to have a lower ability to activate AhR in all donors except Toddler 2,

469 suggesting a potential defect in the microbiota to produce metabolites known to activate this

470 receptor involved in the regulation of intestinal homeostasis [40,62]. Finally, an additional

471 analysis was performed on the microbial volatolome from the luminal phase (**Fig. 7**). 184 VOCs

472 were detected in control and exposed samples (data not shown). The 26 discriminating VOCs

473 between the two groups (**Table 2**) are hydrocarbons and oxygenated compounds (alcohols,

474 aldehydes, ketones and esters). Volatolomic profiles based on these VOCs indicated a profound

475 effect of exposure to PE MPs (**Fig. 7a**). The abundances of the 8 most discriminant VOCs (*p*

476 0.001) are shown in **Table 2** and in **Fig. 7b.** In particular, the 6 hydrocarbon compounds

477 (pentane, 3-methyl-; cyclopentane, 1,1,3-trimethyl-; 1-heptene, 2,4-dimethyl-; heptane, 2,3-

478 dimethyl-; methyl C9-alkane - RI 814, methyl C9-alkane - RI 821 and methyl C9-alkane - RI

479 861), two heptanone (2-heptanone and 2-heptanone, 4-methyl-), two alcohols (1-hexanol and

480 1-heptanol) and two aldehydes (heptanal and octanal) were overproduced while 8 out of 12

481 esters (among which propanoic acid, ethyl ester and butanoic acid) were underproduced after

482 exposure to PE MPs.

3.4 Impact of exposure to PE MPs on intestinal epithelium inflammation, permeability and mucin synthesis as mediated by gut microbiota metabolites

485 In order to explore whether alterations in the metabolic activities of the gut microbiota induced

486 by exposure to PE MPs have consequences on the intestinal barrier, we determined the impact

487 of Tm-ARCOL luminal supernatants on Caco2/HT29-MTX cells. The preliminary toxicity

488 assay on intestinal cells with diluted Tm-ARCOL luminal supernatants showed no cytotoxicity

489 for 1/20 and 1/50 dilutions (data not shown). Therefore, in the following cell experiments, the

490 1/20 (v/v) dilution was used. Various parameters were measured to assess the impact of Tm-

491 ARCOL luminal supernatants on trans- and para-cellular permeability. Gene expression of tight

492 junction proteins, *OCLN* and *ZO-1*, was not significantly altered by Tm-ARCOL luminal

493 supernatants of the exposed microbiota in Tm-ARCOL, although a clear decreasing trend was

494 observed for both genes in Toddler 1 (**Fig. S4a**). Trans-epithelial electric resistance (ΔTEER)

495 (**Fig. 8a**) as well as the translocation of Lucifer yellow (LY) (**Fig. 8b**) and horseradish

496 peroxydase (HRP) (**Fig. 8b**), as indicators of para- and trans-cellular permeability, respectively,

497 were unaffected by Tm-ARCOL luminal supernatants of the exposed microbiota. Interestingly,

498 however, HRP and LY translocation tended to increase in Toddler 1, consistent with decreased

499 *ZO-1* and *OCLN* gene expression. Finally, the expression of mucin-related genes, *MUC2* and

500 *MUC5AC*, was measured without demonstrating any significant effect of Tm-ARCOL luminal

501   supernatants of the exposed microbiota (**Fig. S4b**). Nevertheless, *MUC2* gene expression

502   tended to increase in 3 of the 4 donors (not detected in Toddler 4), while *MUC5AC* gene

503   expression appeared to be slightly overexpressed in Toddlers 1 and 3 only. The inflammatory

504   marker IL-8 (**Fig. 8c**) was also measured and the levels (70-100 pg/mL), detected in the apical

505   and basolateral compartments, were not significantly impacted by the Tm-ARCOL luminal

506   supernatants of the exposed microbiota.

507   **4. Discussion**

508   Ingestion of MPs is increasingly recognized as a threat for human health. However, to date

509   available data on the behavior of MPs throughout the human digestive tract are very limited,

510   especially in the infant population. In this study, we showed for the first time, using an *in vitro*

511   colonic model of toddler, that oral exposure to PE MPs alters the composition of gut microbiota

512   by increasing potentially pathogenic populations of *Dethiosulfovibrionaceae*,

513   *Enterobacteriaceae*, *Oscillospiraceae* and *Moraxellaceae* while decreasing *Monoglobaceae*.

514   These changes in microbial populations have been associated with alterations in metabolic

515   activity, as shown by altered VOC profiles and decreased butyrate production.

516   As an alternative to clinical trials, most of the few studies on the fate of MPs under human

517   simulated digestive conditions have been conducted in *in vitro* models of the upper

518   gastrointestinal tract, i.e. in gastric and small intestinal systems [63–66]. These studies

519   demonstrated that artificial digestive fluids were not able to degrade MPs of five different

520   polymers types (including PE) into smaller fragments or change their shape, size or texture, but

521   described the formation of a biomolecular corona onto the surface of the particle surface

522   [63,64]. Given this lack of physicochemical degradation of MPs in the upper gastrointestinal

523   tract, in the present study we focused on interactions in the lower gut between PE MPs and

524   microbiota, another major component of human gut homeostasis. Yet, only Tamargo and

525   colleagues have studied interactions between human-derived gut microbiota and MPs, but using

526 polyethylene terephthalate -PET- and not PE, also of different size and shape (160 ± 110 μm,

527 irregular morphology) [66]. We also recently performed a study in the Mucosal Artificial Colon

528 (M-ARCOL) where we assessed the impact of repeated exposure to PE MPs on the composition

529 and activity of the adult gut microbiota [44]. Here, as an extension of this work and in order to

530 be as close as possible to the human situation, we have chosen to evaluate in Tm-ARCOL the

531 effect of a physiological dose of MPs at 21 mg/day according to recent exposure data [67]. PE

532 MPs were administrated daily for 14 days to reproduce chronic exposure, while Tamargo and

533 colleagues evaluated the effect of a single dose of 166 mg PET MPs for 72 h. Finally, their

534 study was carried out in adult digestive conditions whereas we have focused here on toddlers,

535 due to the specificities of their digestive physiology and gut microbiota, which makes them a

536 high-risk population regarding the ingestion of xenobiotics [13,26,68]. During the critical

537 period of the first 1000 days, the gut microbiota of infants remains immature in terms of

538 composition and functionalities [35]. This microbiota immaturity has been successfully

539 reproduced in the Tm-ARCOL model [46], with lower microbial diversity, higher abundance

540 of infant-related microbial populations (e.g. *Bifidobacteriaceae*, *Enterobacteriaceaea*), higher

541 acetate production and lower butyrate production in toddlers than in adults, consistent with *in*

542 *vivo* data [69–71]. Repeated addition of PE MPs resulted in an increased α-diversity in Tm-

543 ARCOL, as previously reported in mouse fecal microbiota by Li and colleagues after 5-week

544 exposure to MP PEs of 10-150 μm in size [72]. This could be explained by the fact that PE MPs

545 can provide a surface for the formation of biofilm-like microbial communities, as observed here

546 by scanning electron microscopy and previously described with PET MPs in another human

547 colon model [66]. The presence of MPs as providers of adhesion sites but also new carbon

548 sources for microbes has been already described in an environmental context [73–75]. The

549 second option does not seem feasible here since no degradation of MPs was observed using

550 Raman spectroscopy analysis. In contrast to our results, Tamargo and colleagues reported a loss

551   of observed ASVs in the transverse and descending colonic compartments following a single

552   addition of PET MPs [66], suggesting that the effect of MPs on human gut microbiota diversity

553   would be, in addition to the exposure scenario, plastic-dependent (i.e. polymer type, size,

554   shape). In our previous study of the adult microbiota, no change in bacterial diversity was

555   observed after exposure to PE MPs, suggesting that the immature infant gut microbiota may be

556   more sensitive to MPs than the mature microbiota [44]. When mice were exposed to PE MPs,

557   the microbial shifts observed in fecal samples were study-dependent and not comparable to the

558   changes observed in Tm-ARCOL [72,76], certainly due to the large discrepancies between

559   human and rodent digestion and microbiome [45]. Interestingly, the human *in vitro* study by

560   Tamargo and colleagues [66] on the exposure to PET MPs showed an increase in

561   *Proteobacteria* and *Synergistetes* (phylum of *Dethiosulfovibrionaceae*) as observed in our

562   study, suggesting that these populations could be considered as markers of exposure to MPs in

563   the human gut microbiota. This hypothesis is reinforced by our recent results on the effect of

564   PE MPs on the adult gut microbiota showing an increase, both in the lumen and mucus-

565   associated microbiota, of *Enterobacteriaceae* and *Dethiosulfovibrionaceae* [44]. For the first

566   time, we also showed in Tm-ARCOL that the impact of MPs can be different between the lumen

567   and mucus-associated microbiota. This is particularly interesting as mucosal microbes appear

568   to play an increasing role in human health and disease [77]. In addition, the size of the MPs

569   seems to have a major role on gut microbiota shifts. The use of beads of the same size as in our

570   study (1 to 10 μm) but coming from a different polymer (PS and polyvinyl chloride –PVC-)

571   [78–80] induced more similar effects on mouse fecal microbes (e.g. increase in

572   *Enterobacteriaceae*) than larger PE particles [72,76]. Among the aforementioned cited

573   populations associated to MP exposure, some families, such as *Dethiosulfovibrionaceae* and

574   *Enterobacteriaceae*, are considered as pathobionts. The *Dethiosulfovibrionaceae* population

575   has been linked to colorectal cancer [81], while *Enterobacteriaceae* containing well known

576   enteric pathogens, such as *Escherichia*, *Shigella*, *Campylobacter* or *Salmonella*, may be

577   increased in irritable bowel syndrome -IBS- [82] and intestinal inflammatory diseases in

578   humans [83]. Obviously, not all species in these families are pathogenic but most of them are

579   commensal, highlighting the critical need to better characterize their potential pathogenic

580   characteristics at a lower taxonomic level. It should be noted that the *Enterobacteriaceae*

581   population appears to have a particular affinity for PE MPs. Indeed, *Escherichia coli* adheres

582   to the surface of PE MPs within 30 s [84]. Similarly, in seawater, *Alpha* and

583   *Gammaproteobacteria* have been identified as the core microbiome of biofilms associated with

584   PE MPs [85].

585   Concerning the impact of exposure to PE MPs on the activity of the gut microbiota, apart from

586   our recent study on the adult microbiota in M-ARCOL [44], no *in vitro* or *in vivo* study

587   involving MPs has been conducted to date. Here, a decrease in butyrate production was notably

588   observed following exposure to PE MPs. This has not been previously shown under adult

589   colonic conditions [44], suggesting an infant-specific effect, which may be associated in this

590   at-risk population with deleterious effects. In particular, the increase in butyrate production

591   during infancy has been described as a key health milestone [69] as the effect of high

592   concentrations of fecal butyrate in one-year old child has been shown to protect against allergies

593   and asthma [86,87]. Of note, AhR activity also tended to be reduced following exposure to PE

594   MPs, suggesting less production of AhR receptor ligands by the gut microbiota. This could be

595   related to the decrease in abundance of some *Tannarellaceae*, like *Parabacteroides distasonis*,

596   which are known to be producers of AhR ligands [88]. The ability of the gut microbiota to

597   produce tryptophan-based AhR ligands influences the integrity of the gut epithelium as well as

598   the development, function, production, and maintenance of several key mucosal immune cells

599   and mediators [40,62,89,90]. A deficit in the production of AhR ligands by the microbiota has

600   been reported in inflammatory bowel disease (IBD), celiac disease and obese patients compared

601 to healthy subjects [58,91,92]. As a novel approach, the activity of the gut microbiota was

602 finally investigated in the present study by measuring VOCs. These compounds have important

603 roles in biological interactions between organisms and are currently used as biomarkers of

604 various human diseases, including cancer and gastrointestinal metabolic disorders [93,94].

605 Marked differences between the abundances of VOCs before and after exposure to PE MPs

606 were highlighted, characterized in particular by an underproduction of 8 ester compounds and

607 an overproduction of 7 hydrocarbons. Again, the shifts in VOC profiles under infant colonic

608 conditions were markedly different from those previously obtained under adult conditions [44].

609 An increase in fecal esters has been observed in obese patients with non-alcoholic fatty liver

610 disease [39], in IBS patients [95], but also after acute exposure *in vitro* of human faecal

611 microbiota to benzo[*a*]pyrene [59]. This suggests that overproduction of esters could be an

612 indicator of disturbances in the gut microbiota. Moreover, when detected in the breath, the

613 formation of hydrocarbons/alkanes has advocated as a non-invasive marker of alcoholic

614 hepatitis but also as a measure of lipid peroxidation observed repeatedly in the pathophysiology

615 of IBD [96]. Our study also showed that among other modified VOCs, heptanone (2-heptanone

616 and 2-heptanone, 4-methyl), two alcohols (1-hexanol and 1-heptanol) and two aldehydes

617 (heptanal and octanal) were overproduced after exposure to PE MPs. These compounds were

618 identified during low-density PE photodegradation in the environment [97], suggesting the

619 possibility of PE degradation during our Tm-ARCOL fermentations, but again not consistent

620 with Raman spectroscopy results. In light of these findings, the potential degradation of PE

621 MPs by the human gut microbiota merits further investigation, although the restricted contact

622 time in the human gut during gastrointestinal transit certainly does not promote plastic

623 degradation, compared to longer exposure to soil or water microorganisms in the environment

624 [98–100].

625 Considering the host response to exposure to PE MPs, we finally combined our *in vitro* colon

626 model with co-cultured intestinal epithelial cells. Such a coupling has already been carried out

627 to evaluate the cellular toxicity of virgin or digested PS or PE MPs [44,64] in adult but never

628 in infant digestive conditions. Previous studies in mice orally exposed to PE MPs have reported

629 deleterious effects on the intestinal barrier characterized by a low-grade inflammatory state

630 [72,101] and conflicting results regarding the production of mucus [76,101]. This negative

631 impact of MPs on the intestinal barrier was confirmed *in vitro*. Direct exposure of human

632 intestinal Caco-2 cells to MPs led to oxidative stress for PE MPs [102] and increased epithelial

633 permeability for PS MPs [64]. Here, for the first time under infant conditions, we investigated

634 the potential indirect effect of exposure to PE MPs on human intestinal Caco-2/HT29-MTX

635 cells, i.e. mediated by the metabolites of gut microbes. No significant effect was demonstrated,

636 since we only observed for some toddlers a trend towards an increase in the expression of mucin

637 genes as well as a non-significant decrease in the expression of genes encoding for tight junction

638 proteins (occludin and ZO-1). Overall, this absence of significant deleterious effects suggests a

639 mucus-protective effect of our co-culture model [103] and/or a loss of the putative response

640 triggered by gut microbial metabolites on inflammation and disruption of the intestinal barrier

641 due to the dilution of the Tm-ARCOL luminal supernatants.

642 Taken together, our results suggest that chronic exposure to PE MPs induced significant

643 perturbations in the composition and activity of toddlergut microbiota that may be related to a

644 "dysbiotic" state of the microbiota, even though increased bacterial diversity does not fit the

645 accepted definition of dysbiosis [104]. This study also highlighted that the impact of PE MPs

646 on the gut microbiota and the intestinal barrier *in vitro* was highly dependent on the donor. For

647 example, exposure to MPs had no significant effect on the microbial diversity of Toddler 4 and

648 induced greater deleterious effects on intestinal permeability in Toddler 1 than in the others.

649 Such data could help to define which infants are high and low "responders" to ingesting MPs.

Our *in vitro* approach can definitively help for a better mechanistic understanding of the interactions between ingested micro- or even nano-sized plastics, the gut microbiota and mucus, considering inter-individual specificities. Further studies are needed to better investigate the effects of different plastic types, surface properties and sizes [105–108] on the structure and functions of the gut microbiota. The potential of the Tm-ARCOL model should be also extended to the simulation of other specific at-risk populations, such as neonates or pathological situations associated with gut microbial dysbiosis, such as obesity in infants [109]. Finally, since MPs may be the carrier of various chemical pollutants, heavy metals, antibiotics or pathogens [110–112], their bioavailability and/or their survival and biological effects could be studied using our *in vitro* approach, as well as their interactions with the human gut microbiota.

675

Financial interests

677 The authors declare that the research was conducted in the absence of any commercial or

678 financial relationships that could be considered as a potential conflict of interest.

679

Data Availability Statement

681 Raw data are available at NCBI under the Sequence Read Archive database in the BioProject

682 n° PRJNA831093.

683

Ethics approval

685 This study is a non-interventional study with no additions to usual clinical cares. The protocol

686 does not require approval from an ethics committee according to the French Public Health Law

687 (CSP Art L 1121-1.1).

688

689

690

691 **Tables**

692

693 **Table 1. Primers used for qPCR for total bacteria load, Illumina sequencing for bacteria**
694 **and Archaeal populations and RT-qPCR to monitor intestinal gene expression**

695

Gene	Primer sequence 5'-3'	Target	Annealing temperature (°C)	References
Primers qPCR				
16S	F-GTATTACCGCGGCTGCTG R-ACTCCTACGGGAGGCAG	Total bacteria	58	Yu et al. 2005-
Primers 16S for Illumina sequencing				
16S	F-CCTACGGGNGGCWGCAG R-GACTACHVGGGTATCTAATCC	Bacteria		Klindworth et al. 2013
16S archaea	F-GYGCASCAGKCGMGAAW R-GGACTACVSGGGTATCTAAT	Archaea	-	Takai et Horikoshi, 2000
Primers RT qPCR				
Gene	**Primer sequence 5'-3'**		**Annealing temperature (°C)**	**References**
GAPDH	F-GTGGAAGGACTCATGACCACAG R-GCAGGGATGATGTTCTGGAGAG	Housekeeping gene	60	This study
OCLN	F-CTGAAGCAAGTGAAGGGATCTG R-CCAACCATCTTCTTGATGTGTGA	Occludin	60	This study
ZO-1	F-AGCACAGTGCCTAAAGCTATTCC R-AAGATGGTCATACTGTGGTGGC	Zonula occludens 1	60	This study
MUC2	F-CAGTGCAGCATCCTCAAA R-GCAGAAGCACTCACAGTC	Mucin 2	60	This study
MUC5AC	F-CACACATGGGAAGCTGAG R-TCGGCAGTCAAAGAACAC	Mucin 5AC	60	This study

696

697 **Table 2. Volatile organic compounds significantly altered by chronic exposure to PE MPs**
698 **in Tm-ARCOL**
699 Values are the mean of abundances ($\times 10^5$) of each candidate marker with its standard deviation
700 (in brackets).

Candidate markers	Tentatively identification[a]	m/z[b]	Exp. RI[c]	Ref. RI[d]	CAS number	p value	Control	Exposed	Under/over production compared to the control
Hydrocarbons									
Pentane, 3-methyl-	MS+RI	71	561	578	96-14-0	***	0.2 (74%)	3.9 (29%)	↗
Cyclopentane, 1,1,3-trimethyl-	MS	97	728	NA	4516-69-2	***	0.2 (51%)	3.3 (34%)	↗
Methyl C9-alkane - RI 814	MS	85	814	NA		***	0.5 (49%)	4.8 (22%)	↗
Methyl C9-alkane - RI 821	MS	71	821	NA		***	3.3 (42%)	17.6 (15%)	↗
1-heptene, 2,4-dimethyl-	MS+RI	70	841	855	19549-87-2	***	5.7 (34%)	23.9 (9%)	↗
Heptane, 2,3-dimethyl-	MS+RI	84	856	850	3074-71-3	***	0.7 (44%)	2.1 (17%)	↗
Methyl C9-alkane - RI 861	MS	71	861	NA		*	1.5 (59%)	5.1 (35%)	↗
Esters									
Propanoic acid, ethyl ester	MS+RI	57	710	708	105-37-3	***	1310.7 (26%)	278.9 (57%)	↘
Propanoic acid, 2-methyl-, ethyl ester	MS+RI	71	757	754	97-62-1	*	15.9 (45%)	4.9 (44%)	↘
Butanoic acid, ethyl ester	MS+RI	88	799	802	105-54-4	***	88.4 (15%)	20.1 (59%)	↘
Propanoic acid, propyl ester	MS+RI	57	807	804	106-36-5	**	203.7 (31%)	42.9 (55%)	↘
Butanoic acid, 3-methyl, ethyl ester	MS+RI	88	851	854	108-64-5	**	1.9 (36%)	0.6 (32%)	↘
Butanoic acid, propyl ester	MS+RI	89	892	899	105-66-8	**	31.4 (37%)	8.7 (30%)	↘
Propanoic acid, butyl ester	MS+RI	75	903	908	590-01-2	**	6.0 (3%)	3.7 (29%)	↘
Hexanoic acid, methyl ester	MS+RI	74	923	924	106-70-7	*	3.3 (63%)	26.1 (64%)	↗
Pentanoic acid, propyl ester	MS	71	1006	NA	141-06-0	**	6.2 (17%)	2.5 (41%)	↘
Heptanoic acid, methyl ester	MS+RI	74	1031	1021	106-73-0	*	0.3 (44%)	10.6 (65%)	↗
Heptanoic acid, ethyl ester	MS+RI	88	1093	1097	106-30-9	*	0.5 (67%)	15.1 (74%)	↗
Butanoic acid, hexyl ester	MS+RI	71	1193	1192	2639-63-6	**	0.8 (28%)	1.6 (23%)	↗
Alcohols									
1-hexanol	MS+RI	69	866	867	111-27-3	*	759.7 (27%)	2425.3 (50%)	↗
1-heptanol	MS+RI	70	979	970	111-70-6	**	127.8 (47%	1644.9 (36%)	↗
Aldehydes									
Heptanal	MS+RI	81	900	899	111-71-7	**	6.0 (31%)	14.0 (26%)	↗
Octanal	MS+RI	84	1017	1003	124-13-0	**	4.0 (25%)	9.1 (25%)	↗
Ketones									
2-heptanone	MS+RI	58	887	891	110-43-0	*	17.5 (51%)	75.7 (59%)	↗
2-heptanone, 4-methyl-	MS+RI	58	942	936	6137-06-0	*	2.5 (35%)	4.4 (26%)	↗
2-decanone	MS+RI	58	1197	1192	693-54-9	**	5.6 (16%)	2.5 (28%)	↘

701
702

703 *[a] MS + RI, mass spectrum and RI agree with literature data; MS, mass spectrum agrees with literature*
704 *data; [b] Mass fragment used for area determination; [c,d] Retention indices on a DB5 capillary column*
705 *from experimental run [c] or bibliographic data [d]; NA: Non-available; ↘: underproduction compared*
706 *to the control; ↗: overproduction compared to the control; *p < 0.05; **p < 0.01; ***p < Bonferroni*
707 *corrected values*

Figure captions

710 **Fig. 1. Outline of *in vitro* fermentations and cell culture experiments**

711 **a**) Schematic representation of the Tm-ARCOL gut model and the parameters used to reproduce
712 the toddler colonic conditions. **b**) Tm-ARCOL experimental design and analysis. Four
713 bioreactors inoculated with fecal samples from four different toddlers were run in parallel. After
714 a stabilization phase, PE MPs were added daily into the bioreactor at a dose of 21 mg for 14
715 days. The last three days of the stabilization period (days 6, 7, 8) were chosen as the control
716 while the last three days of the treatment period (days 20, 21, 22) were selected as exposed
717 days. Atmospheric and luminal fermentation media were sampled daily while mucin-alginate
718 beads were collected every other day for further analysis of microbiota structure or activity. **c**)
719 Cell culture experimental design and analysis. Caco-2 and HT29-MTX intestinal cells in co-
720 culture were seeded using inserts. After an 18-day growth period, cells were exposed during 24
721 h to day 8 or day 22 luminal supernatants of Tm-ARCOL and cytotoxicity, inflammation, mucin
722 gene expression and intestinal permeability were evaluated.

723 *AhR: Aryl Hydrocarbon Receptor; ELISA: Enzyme-Linked Immunosorbent Assay; GC: Gas*
724 *Chromatography; HRP: Horse Radish Peroxidase; HPLC: High-Performance Liquid*
725 *Chromatography; LDH: Lactate Dehydrogenase; LY: Lucifer Yellow; MP: Microplastic;*
726 *MUC2: Mucin 2; MUC5AC: Mucin 5AC; OCLN: Occludin; PE: Polyethylene; SCFA: Short*
727 *Chain Fatty Acid; TEER: Trans-Epithelial Electrical Resistance; ZO-1: Zonula Occludens-1*

728

729 **Fig 2. Scanning electron microscopy observations of PE MPs/microbiota interactions in**
730 **Tm-ARCOL and Raman spectroscopy analysis of PE MPs at the end of exposed period**

731 **a**) Representative scanning electron microscopy images of luminal samples and mucin-alginate
732 beads from Toddlers 2 and 3 before and after exposure to PE MPs. For the mucin-alginate
733 beads, the analysis was performed after 48 h of colonization in the mucosal external
734 compartment. The right panel shows a typical mucin-alginate bead and its colonization by gut
735 microbiota (day 8). **b**) Example of Raman spectroscopy mapping of the surface of PE MP (the
736 PE MP appears in yellow in the left image) in the day 22 luminal sample of Toddler 3; the
737 Raman spectrum is compared with that obtained with the initial suspension of PE MPs.

738 *PE: Polyethylene*

739

740 **Fig 3. Effect of chronic exposure to PE MPs on luminal and mucosal bacteria in Tm-**
741 **ARCOL**

742 **a**) Relative abundance of the 20 main bacterial populations at the family level during the control
743 and exposed periods for each donor. M represents the medium surrounding the mucin-alginate
744 beads. **b**) Differential Analysis (DESeq2, metagenomeSeq, metacoder, $p<0.05$) at the family
745 level between control and exposed days (n=4).

746

747 **Fig 4. Effect of chronic exposure to PE MPs on α-and β-diversity indexes of bacterial**
748 **communities in Tm-ARCOL**

Observed ASVs (**a**) and Shannon (**b**) indexes for each donor and on average (n=4), expressed as means ± SEM. Bacterial β-diversity (**c**) was also represented using a two-dimensional PCoA plot, based on the unweighted UniFrac distance matrix on luminal and mucosal samples (left) and a distance-based redundancy analysis (RDA), based on both 16S rDNA gene metabarcoding and metabolic activity data, applied to luminal samples and excluding the donor effect (right). Statistical differences were tested by one-way ANOVA with * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

CONT: Control, EXP: Exposed

Fig 5. Impact of chronic exposure to PE MPs on gas and short chain fatty acid production in Tm-ARCOL

a) Mean ± SEM daily gas production in mL and (**b**) mean ± SEM total SCFA production in mM, for each donor and in average (n=4). **c**) Mean ± SEM daily acetate, propionate and butyrate production in mM for each donor and on average (n=4). Statistical differences were tested by one-way ANOVA with * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

CONT: Control; EXP: Exposed; SCFA: Short Chain Fatty Acid

Fig 6. Impact of chronic exposure to PE MPs on the capacity of Tm-ARCOL luminal supernatants to activate Aryl Hydrocarbon receptor

AhR activity is expressed as fold changes relative to the luciferase activity of the control period and after normalisation based on sample cytotoxicity (lactate dehydrogenase activity assay). All results are given for each donor and on average (n=4) and expressed as means ± SEM.

AhR: Aryl Hydrocarbon Receptor; CONT: Control; EXP: Exposed

Fig 7. Impact of chronic exposure to PE MPs on volatile organic compounds in the luminal compartment of Tm-ARCOL

a) Principal component analysis (PCA) of the control and exposed days including the 26 discriminant VOCs. **b**) Mean of abundance of the eight VOCs with the highest discriminating power. Abundances of selected VOCs are expressed in arbitrary units. *** $p<0.01$ Bonferroni corrected value.

Fig 8. Impact of Tm-ARCOL luminal supernatants before and after chronic exposure to PE MPs on inflammation and intestinal barrier integrity using a Caco-2/HT29-MTX co-culture

a) The difference between the average TEER before and after 24-h exposure to Tm-ARCOL luminal supernatants was calculated and termed ΔTEER (Ω.cm²) as an indicator of paracellular permeability of the co-culture before and after exposure to MPs. **b**) LY transport (Papp in cm/s) and HRP Papp (in cm/s) as an indicator of paracellular and transcellular permeability, respectively. **c**) Mean IL-8 concentration (in pg/mL) in the apical and basolateral media of the cell co-culture. All results were given by donors, also on average (n=4) and expressed as means ± SEM.

CONT: Control; EXP: Exposed; HRP: Horse Radish Peroxidase; IL-8: Interleukin-8; LY: Lucifer Yellow; Papp: Apparent Permeability coefficient; TEER: Trans-Epithelial Electrical Resistance

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