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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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Abstract

Infants are characterized by an immaturity of the gut ecosystem and a high exposure to microplastics (MPs) through diet, dust and suckling. However, the bidirectional interactions between MPs and immature infant intestinal microbiota remain unknown. Our study aims to investigate the impact of chronic exposure to polyethylene (PE) MPs on the gut microbiota and intestinal barrier of infants, using the new Toddler mucosal Artificial Colon coupled with a co-culture of epithelial and mucus-secreting cells. Gut microbiota composition was determined by 16S metabarcoding and microbial activities were evaluated by gas, short chain fatty acid and volatolomics analyses. Gut barrier integrity was assessed *via* evaluation of intestinal permeability, inflammation and mucus synthesis. Exposure to PE MPs induced gut microbial shifts increasing α-diversity and abundance of potentially harmful pathobionts, such as *Dethiosulfovibrionaceae* and *Enterobacteriaceae*. Those changes were associated to butyrate production decrease and major changes in volatile organic compounds profiles. In contrast, no significant impact of PE MPs on the gut barrier, as mediated by microbial metabolites, was reported. For the first time, this study indicates that ingestion of PE MPs can induce perturbations in the gut microbiome of infants. Next step would be to further investigate the potential vector effect of MPs.

Environmental implication

Plastic production has exploded the last half-century, resulting in environmental pollution and microplastics (MPs) contamination throughout the food chain. This raised concerns for human health, particularly in the highly exposed and at-risk infant population. We investigated for the first time the effects of a chronic exposure to polyethylene MPs on immature infant gut microbiome and intestinal barrier, using a novel approach combining a newly developed infant colon model with co-cultures of enterocytes and goblet cells. This work provides useful data

on MP interactions with infant microbiota and mucus, considering inter-individualities, increasing our knowledge on the health impact of MP pollution.

Keywords

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

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

spontaneous abortion [31]. Oral exposure to PS MPs also induced fecal microbiota dysbiosis and gut barrier dysfunction in dams while offspring showed a higher risk of metabolic disorders [32,33]. Importantly, no studies to date have investigated the bidirectional interactions between ingested MPs and the immature and developing infant microbiome, despite its key role in gut homeostasis. Indeed, during the first 1000 days of life, while the digestive physiology is still under development, the infant intestinal microbiota (which mainly colonizes the colonic compartment) simultaneously undergoes significant maturation in terms of composition and metabolic activity [34–36]. The gut microbiome acts primarily in the degradation of undigested food, resulting in the production of secondary metabolites such as gases, short-chain fatty acids (SCFAs) [37,38], volatile organic compounds (VOCs) [39] and aryl hydrocarbon receptor (AhR) ligands [40]. SCFAs (i.e., butyrate, propionate and acetate) are byproducts of the fermentation of nondigestible dietary fibers under the action of intestinal bacteria, whereas AhR ligands are primarily derived from the metabolism of tryptophan. Both groups of metabolites are involved in maintaining the integrity of intercellular tight junction in the intestinal epithelium, thus linking gut microbial activity to the maintenance of effective intestinal barrier function. In addition, the gut microbiota plays an important role in the formation of host immunity, in the regulation of gut endocrine and neurological functions, and together with the intestinal mucus and epithelium, it forms a protective barrier against exogenous threats such as pathogens and foreign particles [41–43].

In this context, as an extension of a previous study by us on adult microbiota [44], we investigated for the first time the impact of oral exposure to PE MPs on the infant microbiome and the intestinal epithelium/mucus barrier. Due to major ethical constraints linked to clinical studies on young populations, but also the striking differences between human and rodent digestive physiology -including microbiota- [45], these experiments were carried out using an innovative *in vitro* approach, combining the Toddler mucosal Artificial Colon (Tm-ARCOL,

[46]) simulating the colonic ecosystem specific to infants (6 months to 3 years) and a co-culture of intestinal epithelial and mucus-secreting goblet cells (Caco-2 and HT29-MTX cells, respectively). After a 2-week daily exposure to PE MPs, the structure and metabolic activities of the infant microbiome were determined by 16S metabarcoding and measurements of gas/SCFAs/VOCs/AhR activity, respectively. Fecal water was then applied to intestinal cell co-cultures to assess the impact of gut microbe metabolites on cell-related inflammatory pathways, mucin synthesis and barrier function.

2. Materials and methods

2.1 Microplastics characterization

PE MPs (CPMS-0.96, 1-10 µm – 0.2 g; non-fluorescent particles) were purchased from Cospheric (CA, USA). The initial powder was suspended (2.625 mg/mL) in a solution of sterile deionized water with 0.01% (w/v) Tween 80 before use. Particle size was determined using the smileview software from scanning electron microscopy images captured with the JSM 6060LV microscope (Jeol, Japan). In order to determine the polymeric composition, the powder was analyzed by differential scanning calorimetry (DSC) and by thermogravimetric analysis (TGA). DSC measurements were performed on a Mettler DSC3^{+} device (N.V. Mettler-Toledo S.A., Belgium). The equipment is calibrated with Indium. A few mg of material were placed in an aluminum pan and then subjected to a temperature cycle ranging from 25°C to 200°C under a flow of nitrogen gas. A rate of 10°C/min was applied to heat and cool the material during the experiment. TGA measurements were performed on a Mettler TGA2 device (N.V. Mettler-Toledo S.A., Belgium). The analysis was performed at a heating rate of 10°C/min under a flow of a nitrogen gas up to 800°C.

2.2 Fecal sample collection and treatment

Fecal samples were collected from four healthy infants (two boys aged 30 and 20 months -Toddlers 1 and 3, and two girls aged 25 and 22 months -Toddlers 2 and 4-) were collected. All

donors had no history of antibiotic treatment or probiotic consumption during the month prior sample collection. Immediately after defecation, the fecal samples (or diapers) were transferred to a sterile container placed in an airtight anaerobic box (GENbag anaer gas pack systems, Biomerieux, France), transported at ambient temperature and processed in the laboratory within 6 h. In an anaerobic chamber (COY laboratories, USA), 11 g of each fresh stool sample were suspended into 110 mL of 30 mM sterile sodium phosphate buffer and filtered (500 µm stainless steel sieve).

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

The Tm-ARCOL is a one-stage fermentation system (MiniBio, Applikon, The Netherlands), operated under continuous conditions, which simulates both physicochemical (pH, retention time, ileal effluents supply and anaerobiosis) and microbial conditions (lumen and mucus associated microbiota) encountered in the colon of young infant [46]. This model is composed of a main bioreactor (luminal compartment) connected to an external glass compartment (mucosal compartment) containing mucin-alginate beads (**Fig. 1a**). These beads were prepared with type II porcine stomach mucin (Sigma-Aldrich, USA) and sodium alginate (Sigma-Aldrich, USA), as previously described [47]. The mucin-alginate beads were stored at 4°C before introduction into the hermetically sealed glass compartment (total beads area of 556 cm^2) connected to the bioreactor. Tm-ARCOL was set-up, based on *in vivo* data, to reproduce the average conditions found in the colon of a healthy toddler, as previously described [46]. Briefly, the composition of the ileal effluents was adjusted to reflect the specificities of the infant diet (**Fig. 1a**). The temperature of the main bioreactor and mucin-alginate bead compartment was maintained at 37°C, the average retention time was set at 19 h and the pH was maintained at a constant value of 6.1 by addition of 2M NaOH. After an initial N_2 flush upon introduction of the fecal inoculum, anaerobiosis was maintained inside the bioreactor by the activity of the resident microbiota alone, in order to faithfully reproduce the *in vivo* situation. The mucin-

alginate beads were renewed every two days while maintaining the external compartment in anaerobiosis thanks to a constant flow of CO_2.

2.4 Experimental design and sampling during *in vitro* fermentations

The experimental design and sampling are summarized in **Fig. 1b**. Four Tm-ARCOL bioreactors were inoculated with fecal samples from the four infant donors and operated in parallel. Fermentations were carried out under continuous conditions for 22 days, after a 24-h batch microbial amplification. An eight-day microbiota stabilization phase was applied and the three last days of this period (days 6, 7 and 8) were chosen as control. Then, fourteen days of daily exposure to PE MPs (21 mg of MPs in 8 mL of a solution of deionized water containing 0.01% (w/v) Tween 80) were carried out and the three last days (days 20, 21 and 22) were chosen as exposure days. Samples from the main bioreactors, termed luminal microbiota, were collected daily for microbiome characterization (storage at -80°C) and SCFA analysis (storage at -20°C). Additional samples from the mucin-alginate bead compartment, termed mucosal microbiota, were collected every other day (when mucin-alginate beads were replaced) for microbiota characterization. Beads were washed twice in sterile phosphate buffer saline -PBS- (pH 7.1) and stored at -80°C before downstream analyses. The medium surrounding mucin-alginate beads was also collected at the same times in case of insufficient amounts of DNA on the mucin-alginate beads. Additional luminal fermentation media were collected on day 8 (control) and day 22 (exposure) for cell culture experiments, AhR activity measurement, scanning electron microscopy, Raman spectroscopy and volatolomic analysis. Samples were also collected daily from the atmospheric phase of the bioreactors to verify anaerobic conditions and determine gas composition. The daily extra volume of gas produced by microbial fermentation was also measured using a syringe connected to the gas bag.

2.5 DNA extraction

Genomic DNA was extracted from luminal samples and mucin-alginate beads (or surrounding medium) using the QIAamp Fast DNA Stool Mini Kit (12830-50, Qiagen, Germany) following the manufacturer's instructions with minor adjustments. Prior to DNA extraction, luminal samples were centrifuged (2000 g, 10 min, 4°C) and the pellet was mechanically disrupted using a bead beater (5 min, 20 beat/sec) with 300 mg of sterile glass beads (diameter ranging from 0.1 to 0.6 mm), incubated (70°C, 5 min) and centrifuged (12000 g, 1 min, 4°C). Mucin-alginate beads were subjected to the following modifications prior to DNA extraction: 10 min of incubation with citrate buffer (37°C), as previously described [48], before vortexing (maximal speed, 3 min) and centrifugation (8000 g, 1 min). DNA integrity was verified by agarose gel electrophoresis and Nanodrop 2000 analysis (Thermo Fisher Scientific, USA). DNA quantity was assessed using the Qubit dsDNA Broad Range Assay Kit (Q32851, Invitrogen, USA) with a Qubit 2.0 Fluorometer (Invitrogen, USA). Samples were stored at -20°C before gut microbiota analysis (qPCR and 16S metabarcoding).

2.6 Total bacteria quantification by qPCR

Total bacteria were quantified by qPCR using primers BAC338R and BAC516F with an hybridization temperature set at 58°C [49] (**Table 1**). Real-time PCR assays were performed on a Biorad CFX96TM Real-Time System (Bio-Rad Laboratories, USA) using the Takyon™ Low Rox SYBR® 2X MasterMix blue dTTP kit (B0701, Eurogentec, Belgium). Each reaction was performed in duplicate in a final volume of 10 µL with 5 µL of Master Mix, 0.45 µL of each primer (10 µM), 1 µL of DNA sample (10 ng/µL) and 3.1 µL of ultra-pure water. The amplifications were performed as follows: 1 cycle at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s and 60°C for 1 min. A melting step was added to ensure the specificity of the primers. The standard curve was generated from 10-fold dilutions of bacterial DNA as described in [47].

2.7 16S metabarcoding and data analysis

The bacterial V3-V4 region of the 16S ribosomal DNA (rDNA) was amplified using primers V3_F357_N and V4_R805 for bacterial fraction and Arch349F and Arch806R for methanogenic Archaea. Amplicons were generated using a Fluidigm Access Array followed by high-throughput sequencing on an Illumina MiSeq system performed at the Carver Biotechnology Center of the University of Illinois (Urbana, USA).

Bioinformatics analysis was performed by GeT-Biopuces platform (INSA/Toulouse Biotechnology Institute, Toulouse, France) with R software version 4.1.1 (2021-08-10) and rANOMALY package [50]. The demultiplexed raw sequence data were filtered by quality and the denoising process performed using DADA2 version 1.20.0 [51]. Reads with N bases or low phred quality score (under 2) were eliminated and reads under 100 pb length removed. Decontamination steps were carried out to filter out sequences corresponding to PhiX DNA used as a spike-in control for MiSeq runs and chimeric sequences were filtered out. Taxonomic affiliation of all amplicon sequence variants (ASVs) was performed with idtaxa function from DECIPHER package version 2.20.0 [52] using SILVA release 138 [53] and GTDB bac120_arc122 [54] databases (60% bootstrap cut-off). To improve completeness of taxonomic affiliation, alignments were carried out using BLAST [55] (98% identity and coverage) on representative sequences of unassigned ASVs or assigned with incomplete taxonomy. A phylogenetic tree was constructed based on the representative ASVs sequences using functions from phangorn package version 2.7.1 [56]. α- and β-diversity indexes were calculated using the diversity_alpha_fun function from rANOMALY package [50]. The across-sample microbiota diversity was described using redundancy analysis (RDA) based on Bray-Curtis dissimilarities using vegan R-package version 2.5-7 [57]. The impact of the different parameters analyzed (total gas and SCFA production, gas and SCFA composition, luminal or mucosal sample types,

time and sex of donors) on dissimilarities among groups was evaluated using PERMANOVA permutation tests (999 permutations).

2.8 Gas analysis

The analysis of O_2, N_2, CO_2, CH_4 and H_2 gases produced during the fermentation process in the atmospheric phase of the main bioreactor was performed using a HP 6890 gas chromatograph (Agilent Technologies, USA) coupled to a micro-TCD detector (Agilent Technologies, USA). Two series of columns, Molecular Sieve 5A and Porapack Q (Agilent Technologies, USA) were used. The composition of the gases was determined using calibration curves produced from 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, 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 % N_2).

2.9 Analysis of short chain fatty acid composition

2 mL of each luminal sample was centrifuged (5000 g, 15 min, 4°C) and 900 µL of the supernatant was diluted at 1/10 in 0.04 M H_2SO_4 mobile phase, vortexed and filtered (pore size 0.22 µm). The three major SCFAs (acetate, propionate and butyrate) were quantified by high-performance liquid chromatography (HPLC) (Elite LaChrom, Merck HITACHI, USA) coupled to a DAD diode. The HPLC column (150 x 7.8 mm) contained a negatively charged sulfonic-grafted polystyrene divinylbenzene stationary phase and carried an eluent containing acidified water. Data were obtained and analyzed by the EZChrom Elite software at 204 and 205 nm. SCFAconcentrations were calculated from calibration curves established from solutions of known increasing concentration of acetate, propionate and butyrate (0, 10, 25 and 40 mM).

2.10 Measurement of AhR activity

The AhR activity of Tm-ARCOL luminal supernatants after exposure to PE MPs (day 22) relative to control conditions (day 8) was measured using a luciferase reporter assay method, as previously described [58]. H1L1.1c2 mouse hepatocellular carcinoma cells, containing a

stably integrated dioxin response element-driven firefly luciferase reporter plasmid pGudLuc1.1, were seeded in 96-well plates at 10^5 cells/well in Minimum Essential Medium Eagle Alpha Modification (MEM-α) medium (with 10% (v/v) heat-inactivated fetal calf serum -FCS, 1% (v/v) penicillin/streptomycin, 1% (v/v) Geneticin G418) and cultured (37°C, 5% CO_2) 24 h before stimulation with the Tm-ARCOL supernatants for 24 h. After incubation, the wells were washed with 100 µL PBS (pH 7), and 50 µL of Promega lysis buffer (pH 7.8) was added to each well. The plates were shaken for 1 h for cell lysis. After addition of 100 µL of luciferase reagent (Promega, France), the luciferase activity was measured using a luminometer (Tecan, Switzerland). The experiments were performed in triplicate. All values were normalized for sample cytotoxicity using the Lactate Dehydrogenase Activity Assay (Promega, USA).

2.11 Volatolomic analysis

Volatile organic compounds (VOCs) from control (day 8) and exposed (day 22) luminal samples were analyzed by solid-phase microextraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS), as previously described [59,60]. Briefly, the day before analysis, 2.2 mL of saturated NaCl solution (360 g/L) was added to each sample (0.8 mL). The mixing vials were closed under a N_2 flow, vortexed for homogenization and thawed for 24 h at 4°C. The following steps were then performed with an automated sampler (AOC-5000 Shimadzu, Japan): (i) preheating of the sample to 40°C for 10 min in the shaker (500 rpm), (ii) SPME trapping (75 µm carboxen/polydimethylsiloxane, 23-gauge needle, Supelco, USA) of the VOCs for 30 min at 40°C, and (iii) thermal desorption at 250°C for 2 min in splitless mode in the GC inlet. Further analysis of VOCs was performed by GC/MS-full scan (GC2010, QP2010+, Shimadzu, Japan). VOCs were injected in a DB-5MS capillary column (60 m × 0.32 mm × 1 µm, Agilent, USA) according to the GC-MS parameters used by Defois and collaborators [59,60]. Provisional identification of VOCs was performed on the basis of mass

spectra, by comparison with mass spectra libraries (Wiley Registry 12th Edition / NIST 2020), and of retention indices (RI), by comparison with published RI values and with those of our in-house database. The peak area of the tentatively identified compounds was determined for each of the targeted molecules using a mass fragment selected for its specificity and lack from co-elution.

2.12 Scanning electron microscopy analysis

The luminal and mucosal samples of the Toddlers 2 and 3, one girl and one boy, were deposited on SEMPore filters (Jeol, Japan) and fixed for 12 h at 4°C in 0.2 M sodium cacodylate buffer at pH 7.4 containing 4% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde. The filters were washed for 10 min in sodium cacodylate buffer (0.2 M, pH 7.4) and post-fixed for 1 h with 1% (w/v) osmium tetroxide in the same buffer. The filters were then washed 20 min in distilled water. Gradual ethanol dehydration was performed from 25° to 100° (10 min each) and completed in hexamethyldisilazane (HMDS) for 10 min. The samples were mounted on stubs using adhesive carbon tabs and sputter-coated with gold-palladium (JFC-1300, Jeol, Japan). The analysis was carried out using a scanning electron microscope JSM-6060LV (Jeol, Japan) at 5kV in high vacuum mode.

2.13 Raman spectroscopy mapping

Mapping by Raman spectroscopy was carried out using a Raman spectrometer (Alpha300R Apyron, Witec, Germany) equipped with a confocal microscope. Exposed luminal samples (day 22, Toddler 3) were vacuum filtered using an Anodisc filter (pore size 0.2 µm, diameter 25 mm). The filters were excited with a Coherent sapphire laser (532 nm, laser power: 10 mW) and Raman spectra were recorded in backscattering configuration using a 50X objective (Zeiss, Germany) with a numerical aperture of 0.55 to achieve a theoretical spatial resolution of 590 nm in the focal plane. Individual Raman spectra were recorded 10 times with an integration time of 1 s. For each filter, maps between 900 and 2500 spectra were recorded with a step of

1.33 µm and 2 µm, respectively. For each mapping, the recorded signal (Raman signal plus background) was integrated over a 20 cm^{-1} spectral range of centered at 1294 cm^{-1} corresponding to the CH_2 twisting vibration band of PE. The signal value was then automatically transformed by the software in a color scale (from black to bright yellow).

2.14 Intestinal cell lines and cell culture conditions

The human colon adenocarcinoma Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC, UK) and used at passage 58-62. The mucus-secreting colon adenocarcinoma HT29-MTX cell line was kindly provided by Dr. Thécla Lesuffleur (INSERM Lille, France) and used at passage 10-16. Both cell lines were maintained in cell culture medium (Dulbecco/Vogt modified Eagle's minimal essential medium -DMEM- without phenol red and 4.5 g/L glucose), supplemented with 1% (v/v) penicillin/streptomycin, 1% (v/v) Non-Essential Amino Acids –NEAA-, 1% (v/v) Glutamax, and 10% (v/v) heat-inactivated fetal bovine serum -FBS- at 37°C in a humidified atmosphere of 5% CO_2/95% air. The medium was changed every two to three days and cells were split at 80% of confluence using Tryple express. The intestinal cells (90% Caco-2/10% HT29-MTX) were seeded in 24-well inserts with 1-µm pore size (Millipore, Merck Millipore SAS, France) and maintained in the culture medium. From day 17, FBS of the culture medium was replaced by 1% (v/v) Insulin Transferrin Selenium (ITS) in order to limit any interference with the samples to be tested. After 18 days, Caco-2/HT29-MTX co-cultures were incubated apically for 24 h with Tm-ARCOL luminal samples taken on day 8 (control) and day 22 (exposed), centrifuged (5000 g, 15 min, 4°C) and diluted 20-foldin cell culture medium (**Fig. 1c**). Experiments were performed with 2 biological replicates and 5 technical replicates for each donor.

2.15 Cell viability

In order to determine the potential cytotoxic effect of Tm-ARCOL luminal supernatants on Caco-2/HT29-MTX cells and to establish subtoxic conditions for testing on inserts, different

dilutions (1/2, 1/5, 1/10, 1/20, 1/30 and 1/50 in cell culture medium) were evaluated. Cell viability was measured in triplicate with CytoTox 96® non-radioactive cytotoxicity test (Promega, France) after 24 h of exposure. In 96-well plates, 25 µL of apical cell culture supernatants were diluted in 25 µL of 1% (v/v) ITS culture medium and mixed with 50 µL of substrate. The plates were then incubated for 15 min at room temperature in the dark. The reaction was stopped with 50 µL of stop solution (1M CH_3COOH) and absorbance at 490 nm was measured with a Spark spectrophotometer (Tecan, Switzerland).

2.16 Intestinal permeability

Starting on day 12, trans-epithelial electrical resistance (TEER) was continuously monitored in real time using the CellZScope2 device (NanoAnalytics, Germany). In addition, after a 24-h contact time with the 20-fold diluted luminal supernatants of Tm-ARCOL, the inserts were transferred to a new 24-well plate containing 1 mL of Hank's balanced salt solution -HBSS. Lucifer yellow -LY- (0.4 mg/mL, molecular weight 457 g/mol) and horseradish peroxydase -HRP- (0.4 mg/mL, molecular weight 44,000 g/mol) were dissolved in HBSS and added to the apical compartment. After 2 h at 37°C under an atmosphere of 5% CO_2/95% air, samples were taken from the apical and basolateral compartments. For LY measurement, samples were analyzed in duplicate using a Spark multimode plate reader (Tecan, Switzerland). Excitation and emission wavelengths were 405 nm and 535 nm, respectively. Total HRP was determined by an ELISA assay. Briefly, 96-well black flat-bottom plates (Greiner, Dutcher, France) were coated overnight at 4°C with 50 µL of 10 µg/mL HRP mouse polyclonal (Abcam, France) in PBS. Plates were blocked with PBS-1% bovine serum albumin (BSA) before incubation with apical and basolateral samples collected from inserts. Rabbit polyclonal anti-HRP biotin (Abcam, France) was added at a concentration of 10 µg/mL before adding Fluorescein-5-isothiocyanate -FITC- conjugated streptavidin (Becton Dickinson, France) for 20 min and fluorescence intensity was measured at 485 nm/525 nm using a Spark microplate reader (Tecan,

Switzerland). The apparent permeability coefficient (Papp in cm/s) was determined by the following equation: Papp=Cb/(SxC0), where Cb represents the level of accumulation of LY or HRP in the basolateral compartment after 2 h, S represents the surface of the membrane (0.33 cm^2) and C0 is the initial concentration of the marker (0.4 mg/mL -LY- or -HRP-) in the apical compartment.

2.17 Interleukin-8 level measurement

The apical and basolateral media of the co-cultures were collected after the contact time of 24 h with the 20-fold diluted luminal supernatants of Tm-ARCOL and stored at -20°C until analysis. Interleulin-8 (IL-8) levels were measured using the Quantikine Human IL-8 ELISA kit (DY208-05, R&D Systems, USA) following the manufacturer's instructions. Briefly, in Maxisorp 96-well plates (Nunc, France), biotinylated Goat polyclonal anti IL-8 was added at a concentration of 10 ng/mL before adding HRP-conjugated streptavidin (Becton Dickinson, France) for 20 min. Absorbance intensity was measured at 450 nm and 540 nm using a Spark microplate reader (Tecan, Switzerland).

2.18 Intestinal cell gene expression

Total RNAs were extracted from cells for each insert using the AllPrep RNA Mini kit (Qiagen, France) according to the manufacturer's instructions. Quantity and quality of extracted RNA samples were evaluated using Nanodrop (Nanophotometer Implen-Dutscher, France) and Bioanalyzer (Agilent Technologies, USA), respectively. RNAs were reverse transcribed using enzyme IScript reverse transcription supermix (Biorad, France). Sample concentration was adjusted to 50 ng/µL. 384-well plates were filled by an Agilent Bravo Automated Liquid Handling Platform (Agilent Technologies, France). All wells contained 5 µL of the following mix: 2.5 µL of IQ SYBR green Supermix (Biorad, France), 1.5 µL of each primer set and 1 µL of cDNA sample. Amplification was performed using a ViiA7 Real-Time PCR System (Applied Biosystems-ThermoFisher Scientific, France). Thermal cycling conditions were as

follows: 3 min denaturation at 95°C followed by 40 cycles at 95°C for 15 s and 45 s at 60°C, and a melting curve step. The primer sequences of the targeted genes (genes encoding tight junction (TJ) proteins (*ZO-1*, *OCLN*) and genes involved in mucus synthesis (*MUC2*, *MUC5AC*) are listed in **Table 1**. Raw data that passed quality control were analyzed with LinRegPCR (version 2021.2) and then normalized against the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), a reference housekeeping gene.

2.19 Statistical analysis

Statistical analyses on gut microbiota activity (gas, SCFAs, AhR activity), α-diversity indexes (number of observed ASVs and Shannon index) from metabarcoding data, as well as results from cell culture experiments, obtained in control and exposed groups, were performed using GraphPad Prism software version 9.3.1 for Windows (GraphPad Software, USA). Data normal distribution was verified by combining Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests and homoscedasticity was checked using the Fisher test. Then, appropriate statistical analysis was applied (either one-way ANOVA, Kruskal-Wallis or Welch's tests) and significance was considered for $p<0.05$. For gut microbiota results, differential analyses (DESeq2, metagenomeSeq, metacoder) were performed on ExploreMetabar software using rANOMALY package [50,61]. Principal coordinate analysis (PCoA) of unweight UniFrac distances was performed and significance between groups was assessed with a permutational multivariate analysis of variance (PERMANOVA) using ADONIS (999 permutations) with non-parametric tests. Volatolomics data were processed using the Statistica Software (v.13) (StatSoft, France). Student's t-tests ($p<0.05$) were carried out for the VOC abundances determined in control (day 8) and exposed (day 22) groups and principal component analyses (PCA) were performed on the discriminant VOCs selected to 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

(**Fig. S2b**). These data also highlighted clear differences between lumen and mucus-associated microbiota and, as expected, their stability at the end of the stabilization phase. After 14 days of daily exposure to PE MPs, clear shifts in bacterial populations were observed both in the luminal and mucosal compartments, and both at the phylum (**Fig. S2a**) and family (**Fig. 3a**) levels. Those changes were again donor-dependent. For instance, exposure to PE MPs resulted in disappearance of *Verrucomicrobia* (*Akkermansiaceae*) in Toddler 2 whereas opposite trends were seen in Toddler 4 and *Synergistetes* (*Dethiosulfovibrionaceae*) increased only in Toddler 3. Exposure to PE MPs also induced in the luminal phase a loss in *Ruminococcaceae* in Toddlers 1 and 2 while *Rikenellaceae* and *Sutterellaceae* decreased in Toddlers 2 and 4. In addition, the population of *Tannerellaceae* population, which was only present in Toddler 1, disappeared from the mucosal phase after exposure to MPs whereas *Enterobacteriaceae* increased in mucus-associated bacteria from Toddlers 2 and 4. Differential analysis of gut microbiota composition in all donors confirmed significant changes in bacterial populations. In the luminal phase, abundance of *Dethiosulfovibrionaceae*, *Planococcaceae*, *Enterobacteriaceae*, *Moraxellaceae*, *Acidaminococcaceae* and *Oscillospiraceae* increased significantly (p<0.05, n=4) after exposure to PE MPs, while the proportion of *Tannerellaceae*, *Rikenellaceae* and *Monoglobaceae* decreased (**Fig. 3b**).

Fewer populations were affected in the mucosal compartment, with an increased abundance of *Dethiosulfovibrionaceae*, *Planococcaceae*, *Acidaminococcaceae* and *Oscillospiraceae* but a loss in *Lachnospiraceae* and *Monoglobaceae* after exposure to PE MPs. The impact of such exposure on microbiota structure was confirmed by β-diversity analysis, which showed clustering between control and exposed samples (**Fig. 4c**), with a greater impact in Toddlers 1, 2 and 3 compared to Toddler 4. Accordingly, MP treatment also resulted in a significant increase in observed ASVs (p<0.01) in the luminal microbiota only for Toddlers 1, 2 and 3. Similarly, in mucosal samples, the number of observed ASVs tended to increase in Toddlers 1

and 2, and significance was reached in Toddler 3 (p<0.05). Finally, RDA analysis of bacterial β-diversity, based on both 16S metabarcoding and metabolic activity data (see 3.3), indicated 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

In parallel to microbiota composition, the impact of exposure to PE MPs was also monitored *via* gut microbial activities, first by monitoring fermentation gas and SCFAs daily **(Fig. S3)**. Similarly to the microbiota composition described above, this analysis showed stable gas and SCFA profiles for each donor at the end of the stabilization period, with the exception of Toddler 3 due to technical problems. Exposure to PE MPs had very limited impact on total gas and SCFA production (**Fig. 5a** and **5b**). Regarding the production of major SCFAs (**Fig. 5c**), acetate tended to increase after exposure to PE MPs for Toddler 1, 2 and 3, while the opposite trend was observed for propionate. Butyrate production dropped after exposure to PE MPs for all donors with significance reached only for Toddlers 1 (p<0.0001) and 4 (p<0.05), and when data were averaged (p<0.0001, n=4). The ability of the gut microbiota to produce ligands capable to activate AhR was also determined in Tm-ARCOL supernatants after exposure to PE MPs (day 22) compared to control conditions (day 8) (**Fig. 6**). After exposure to PE MPs, luminal contents tended to have a lower ability to activate AhR in all donors except Toddler 2, suggesting a potential defect in the microbiota to produce metabolites known to activate this receptor involved in the regulation of intestinal homeostasis [40,62]. Finally, an additional analysis was performed on the microbial volatolome from the luminal phase (**Fig. 7**). 184 VOCs were detected in control and exposed samples (data not shown). The 26 discriminating VOCs between the two groups (**Table 2**) are hydrocarbons and oxygenated compounds (alcohols, aldehydes, ketones and esters). Volatolomic profiles based on these VOCs indicated a profound effect of exposure to PE MPs (**Fig. 7a**). The abundances of the 8 most discriminant VOCs (*p*

0.001) are shown in **Table 2** and in **Fig. 7b.** In particular, the 6 hydrocarbon compounds (pentane, 3-methyl-; cyclopentane, 1,1,3-trimethyl-; 1-heptene, 2,4-dimethyl-; heptane, 2,3-dimethyl-; methyl C9-alkane - RI 814, methyl C9-alkane - RI 821 and methyl C9-alkane - RI 861), two heptanone (2-heptanone and 2-heptanone, 4-methyl-), two alcohols (1-hexanol and 1-heptanol) and two aldehydes (heptanal and octanal) were overproduced while 8 out of 12 esters (among which propanoic acid, ethyl ester and butanoic acid) were underproduced after 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

In order to explore whether alterations in the metabolic activities of the gut microbiota induced by exposure to PE MPs have consequences on the intestinal barrier, we determined the impact of Tm-ARCOL luminal supernatants on Caco2/HT29-MTX cells. The preliminary toxicity assay on intestinal cells with diluted Tm-ARCOL luminal supernatants showed no cytotoxicity for 1/20 and 1/50 dilutions (data not shown). Therefore, in the following cell experiments, the 1/20 (v/v) dilution was used. Various parameters were measured to assess the impact of Tm-ARCOL luminal supernatants on trans- and para-cellular permeability. Gene expression of tight junction proteins, *OCLN* and *ZO-1*, was not significantly altered by Tm-ARCOL luminal supernatants of the exposed microbiota in Tm-ARCOL, although a clear decreasing trend was observed for both genes in Toddler 1 (**Fig. S4a**). Trans-epithelial electric resistance (ΔTEER) (**Fig. 8a**) as well as the translocation of Lucifer yellow (LY) (**Fig. 8b**) and horseradish peroxydase (HRP) (**Fig. 8b**), as indicators of para- and trans-cellular permeability, respectively, were unaffected by Tm-ARCOL luminal supernatants of the exposed microbiota. Interestingly, however, HRP and LY translocation tended to increase in Toddler 1, consistent with decreased *ZO-1* and *OCLN* gene expression. Finally, the expression of mucin-related genes, *MUC2* and *MUC5AC*, was measured without demonstrating any significant effect of Tm-ARCOL luminal

supernatants of the exposed microbiota (**Fig. S4b**). Nevertheless, *MUC2* gene expression tended to increase in 3 of the 4 donors (not detected in Toddler 4), while *MUC5AC* gene expression appeared to be slightly overexpressed in Toddlers 1 and 3 only. The inflammatory marker IL-8 (**Fig. 8c**) was also measured and the levels (70-100 pg/mL), detected in the apical and basolateral compartments, were not significantly impacted by the Tm-ARCOL luminal supernatants of the exposed microbiota.

4. Discussion

Ingestion of MPs is increasingly recognized as a threat for human health. However, to date available data on the behavior of MPs throughout the human digestive tract are very limited, especially in the infant population. In this study, we showed for the first time, using an *in vitro* colonic model of toddler, that oral exposure to PE MPs alters the composition of gut microbiota by increasing potentially pathogenic populations of *Dethiosulfovibrionaceae*, *Enterobacteriaceae*, *Oscillospiraceae* and *Moraxellaceae* while decreasing *Monoglobaceae*. These changes in microbial populations have been associated with alterations in metabolic activity, as shown by altered VOC profiles and decreased butyrate production.

As an alternative to clinical trials, most of the few studies on the fate of MPs under human simulated digestive conditions have been conducted in *in vitro* models of the upper gastrointestinal tract, i.e. in gastric and small intestinal systems [63–66]. These studies demonstrated that artificial digestive fluids were not able to degrade MPs of five different polymers types (including PE) into smaller fragments or change their shape, size or texture, but described the formation of a biomolecular corona onto the surface of the particle surface [63,64]. Given this lack of physicochemical degradation of MPs in the upper gastrointestinal tract, in the present study we focused on interactions in the lower gut between PE MPs and microbiota, another major component of human gut homeostasis. Yet, only Tamargo and colleagues have studied interactions between human-derived gut microbiota and MPs, but using

polyethylene terephthalate -PET- and not PE, also of different size and shape (160 ± 110 µm, irregular morphology) [66]. We also recently performed a study in the Mucosal Artificial Colon (M-ARCOL) where we assessed the impact of repeated exposure to PE MPs on the composition and activity of the adult gut microbiota [44]. Here, as an extension of this work and in order to be as close as possible to the human situation, we have chosen to evaluate in Tm-ARCOL the effect of a physiological dose of MPs at 21 mg/day according to recent exposure data [67]. PE MPs were administrated daily for 14 days to reproduce chronic exposure, while Tamargo and colleagues evaluated the effect of a single dose of 166 mg PET MPs for 72 h. Finally, their study was carried out in adult digestive conditions whereas we have focused here on toddlers, due to the specificities of their digestive physiology and gut microbiota, which makes them a high-risk population regarding the ingestion of xenobiotics [13,26,68]. During the critical period of the first 1000 days, the gut microbiota of infants remains immature in terms of composition and functionalities [35]. This microbiota immaturity has been successfully reproduced in the Tm-ARCOL model [46], with lower microbial diversity, higher abundance of infant-related microbial populations (e.g. *Bifidobacteriaceae*, *Enterobacteriaceaea*), higher acetate production and lower butyrate production in toddlers than in adults, consistent with *in vivo* data [69–71]. Repeated addition of PE MPs resulted in an increased α-diversity in Tm-ARCOL, as previously reported in mouse fecal microbiota by Li and colleagues after 5-week exposure to MP PEs of 10-150 µm in size [72]. This could be explained by the fact that PE MPs can provide a surface for the formation of biofilm-like microbial communities, as observed here by scanning electron microscopy and previously described with PET MPs in another human colon model [66]. The presence of MPs as providers of adhesion sites but also new carbon sources for microbes has been already described in an environmental context [73–75]. The second option does not seem feasible here since no degradation of MPs was observed using Raman spectroscopy analysis. In contrast to our results, Tamargo and colleagues reported a loss

of observed ASVs in the transverse and descending colonic compartments following a single addition of PET MPs [66], suggesting that the effect of MPs on human gut microbiota diversity would be, in addition to the exposure scenario, plastic-dependent (i.e. polymer type, size, shape). In our previous study of the adult microbiota, no change in bacterial diversity was observed after exposure to PE MPs, suggesting that the immature infant gut microbiota may be more sensitive to MPs than the mature microbiota [44]. When mice were exposed to PE MPs, the microbial shifts observed in fecal samples were study-dependent and not comparable to the changes observed in Tm-ARCOL [72,76], certainly due to the large discrepancies between human and rodent digestion and microbiome [45]. Interestingly, the human *in vitro* study by Tamargo and colleagues [66] on the exposure to PET MPs showed an increase in *Proteobacteria* and *Synergistetes* (phylum of *Dethiosulfovibrionaceae*) as observed in our study, suggesting that these populations could be considered as markers of exposure to MPs in the human gut microbiota. This hypothesis is reinforced by our recent results on the effect of PE MPs on the adult gut microbiota showing an increase, both in the lumen and mucus-associated microbiota, of *Enterobacteriaceae* and *Dethiosulfovibrionaceae* [44]. For the first time, we also showed in Tm-ARCOL that the impact of MPs can be different between the lumen and mucus-associated microbiota. This is particularly interesting as mucosal microbes appear to play an increasing role in human health and disease [77]. In addition, the size of the MPs seems to have a major role on gut microbiota shifts. The use of beads of the same size as in our study (1 to 10 µm) but coming from a different polymer (PS and polyvinyl chloride –PVC-) [78–80] induced more similar effects on mouse fecal microbes (e.g. increase in *Enterobacteriaceae*) than larger PE particles [72,76]. Among the aforementioned cited populations associated to MP exposure, some families, such as *Dethiosulfovibrionaceae* and *Enterobacteriaceae*, are considered as pathobionts. The *Dethiosulfovibrionaceae* population has been linked to colorectal cancer [81], while *Enterobacteriaceae* containing well known

enteric pathogens, such as *Escherichia*, *Shigella*, *Campylobacter* or *Salmonella,* may be increased in irritable bowel syndrome -IBS- [82] and intestinal inflammatory diseases in humans [83]. Obviously, not all species in these families are pathogenic but most of them are commensal, highlighting the critical need to better characterize their potential pathogenic characteristics at a lower taxonomic level. It should be noted that the *Enterobacteriaceae* population appears to have a particular affinity for PE MPs. Indeed, *Escherichia coli* adheres to the surface of PE MPs within 30 s [84]. Similarly, in seawater, *Alpha* and *Gammaproteobacteria* have been identified as the core microbiome of biofilms associated with PE MPs [85].

Concerning the impact of exposure to PE MPs on the activity of the gut microbiota, apart from our recent study on the adult microbiota in M-ARCOL [44], no *in vitro* or *in vivo* study involving MPs has been conducted to date. Here, a decrease in butyrate production was notably observed following exposure to PE MPs. This has not been previously shown under adult colonic conditions [44], suggesting an infant-specific effect, which may be associated in this at-risk population with deleterious effects. In particular, the increase in butyrate production during infancy has been described as a key health milestone [69] as the effect of high concentrations of fecal butyrate in one-year old child has been shown to protect against allergies and asthma [86,87]. Of note, AhR activity also tended to be reduced following exposure to PE MPs, suggesting less production of AhR receptor ligands by the gut microbiota. This could be related to the decrease in abundance of some *Tannarellaceae*, like *Parabacteroides distasonis*, which are known to be producers of AhR ligands [88]. The ability of the gut microbiota to produce tryptophan-based AhR ligands influences the integrity of the gut epithelium as well as the development, function, production, and maintenance of several key mucosal immune cells and mediators [40,62,89,90]. A deficit in the production of AhR ligands by the microbiota has been reported in inflammatory bowel disease (IBD), celiac disease and obese patients compared

to healthy subjects [58,91,92]. As a novel approach, the activity of the gut microbiota was finally investigated in the present study by measuring VOCs. These compounds have important roles in biological interactions between organisms and are currently used as biomarkers of various human diseases, including cancer and gastrointestinal metabolic disorders [93,94]. Marked differences between the abundances of VOCs before and after exposure to PE MPs were highlighted, characterized in particular by an underproduction of 8 ester compounds and an overproduction of 7 hydrocarbons. Again, the shifts in VOC profiles under infant colonic conditions were markedly different from those previously obtained under adult conditions [44]. An increase in fecal esters has been observed in obese patients with non-alcoholic fatty liver disease [39], in IBS patients [95], but also after acute exposure *in vitro* of human faecal microbiota to benzo[*a*]pyrene [59]. This suggests that overproduction of esters could be an indicator of disturbances in the gut microbiota. Moreover, when detected in the breath, the formation of hydrocarbons/alkanes has advocated as a non-invasive marker of alcoholic hepatitis but also as a measure of lipid peroxidation observed repeatedly in the pathophysiology of IBD [96]. Our study also showed that among other modified VOCs, heptanone (2-heptanone and 2-heptanone, 4-methyl), two alcohols (1-hexanol and 1-heptanol) and two aldehydes (heptanal and octanal) were overproduced after exposure to PE MPs. These compounds were identified during low-density PE photodegradation in the environment [97], suggesting the possibility of PE degradation during our Tm-ARCOL fermentations, but again not consistent with Raman spectroscopy results. In light of these findings, the potential degradation of PE MPs by the human gut microbiota merits further investigation, although the restricted contact time in the human gut during gastrointestinal transit certainly does not promote plastic degradation, compared to longer exposure to soil or water microorganisms in the environment [98–100].

Considering the host response to exposure to PE MPs, we finally combined our *in vitro* colon model with co-cultured intestinal epithelial cells. Such a coupling has already been carried out to evaluate the cellular toxicity of virgin or digested PS or PE MPs [44,64] in adult but never in infant digestive conditions. Previous studies in mice orally exposed to PE MPs have reported deleterious effects on the intestinal barrier characterized by a low-grade inflammatory state [72,101] and conflicting results regarding the production of mucus [76,101]. This negative impact of MPs on the intestinal barrier was confirmed *in vitro*. Direct exposure of human intestinal Caco-2 cells to MPs led to oxidative stress for PE MPs [102] and increased epithelial permeability for PS MPs [64]. Here, for the first time under infant conditions, we investigated the potential indirect effect of exposure to PE MPs on human intestinal Caco-2/HT29-MTX cells, i.e. mediated by the metabolites of gut microbes. No significant effect was demonstrated, since we only observed for some toddlers a trend towards an increase in the expression of mucin genes as well as a non-significant decrease in the expression of genes encoding for tight junction proteins (occludin and ZO-1). Overall, this absence of significant deleterious effects suggests a mucus-protective effect of our co-culture model [103] and/or a loss of the putative response triggered by gut microbial metabolites on inflammation and disruption of the intestinal barrier due to the dilution of the Tm-ARCOL luminal supernatants.

Taken together, our results suggest that chronic exposure to PE MPs induced significant perturbations in the composition and activity of toddlergut microbiota that may be related to a "dysbiotic" state of the microbiota, even though increased bacterial diversity does not fit the accepted definition of dysbiosis [104]. This study also highlighted that the impact of PE MPs on the gut microbiota and the intestinal barrier *in vitro* was highly dependent on the donor. For example, exposure to MPs had no significant effect on the microbial diversity of Toddler 4 and induced greater deleterious effects on intestinal permeability in Toddler 1 than in the others. 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.

Fundings

This work was supported by a grant from the French Ministère de l'Enseignement Supérieur et de la Recherche to EF and UMR MEDIS, and by the National Research Institute for Agriculture, Food and Environment (INRAE, France PlasToX project) and by the French National Research Agency (ANR-19-MRS2-0011 HuPlastiX project) to MMB.

Acknowledgements

The authors thank Etienne Rifa from GeT-Biopuces platform (INSA/TBI, Toulouse, France) for help with bioinformatics analysis, Sandrine Chalancon for technical help on qPCR analysis and Tm-ARCOL experiments. The authors also thank the young donors and their parents for providing fecal materials. The authors are grateful to Frederic Mercier and Nathalie Kondjoyan (MASS Team, INRAE UR370 QuaPA) for volatolomic analyses. The authors thank Christelle Blavignac, Centre Imagerie Cellulaire Santé (CICS) - UCA PARTNER, for her technical support and expertise in microscopy analysis.

Financial interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

Data Availability Statement

Raw data are available at NCBI under the Sequence Read Archive database in the BioProject n° PRJNA831093.

Ethics approval

This study is a non-interventional study with no additions to usual clinical cares. The protocol does not require approval from an ethics committee according to the French Public Health Law (CSP Art L 1121-1.1).

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

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Table 2. Volatile organic compounds significantly altered by chronic exposure to PE MPs in Tm-ARCOL

Values are the mean of abundances ($\times 10^5$) of each candidate marker with its standard deviation (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%)	↘

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

Figure captions

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

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

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

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

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

PE: Polyethylene

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

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

Fig 4. Effect of chronic exposure to PE MPs on α-and β-diversity indexes of bacterial 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 ($\Omega.cm^2$) 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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