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Single exposure of food-derived polyethylene and polystyrene microplastics profoundly affects gut microbiome in an *in vitro* colon model

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ABSTRACT

Microplastics (MPs) are widespread contaminants highly persistent in the environment and present in matrices to which humans are extensively exposed, including food and beverages. MP ingestion occurs in adults and children and is becoming an emerging public health issue. The gastrointestinal system is the most exposed to MP contamination, which can alter its physiology starting from changes in the microbiome. This study investigates by an omic approach the impact of a single intake of a mixture of polyethylene (PE) and polystyrene (PS) MPs on the ecology and metabolic activity of the colon microbiota of healthy volunteers, in an *in vitro* intestinal model. PE and PS MPs were pooled together in a homogeneous mix, digested with the INFOGEST system, and fermented with MICODE (multi-unit *in vitro* colon model) at loads that by literature correspond to the possible intake of food-derived MPs of a single meal. Results demonstrated that MPs induced an opportunistic bacteria overgrowth (*Enterobacteriaceae*, *Desulfovibrio* spp., *Clostridium* group I and *Atopobium* – *Collinsella* group) and a contextual reduction on abundances of all the beneficial taxa analyzed, with the sole exception of *Lactobacillales*. This microbiota shift was consistent with the changes recorded in the bacterial metabolic activity.

1. Introduction

Plastics are versatile materials whose demand has continuously increased since their invention because of low cost, lightweight, waterproof, insulating properties and ease of manufacturing. At the same time, robustness, durability, and resilience within a wide range of temperature make conventional plastics highly persistent in the environment, with extremely low rates of natural removal on the scale of centuries (Ioakeimidis et al., 2016). Moreover, well before degradation weathering processes cause fragmentation of plastic items into microplastics (MPs; <1 mm) and nanoplastics (NPs; < 1 μm) (EFSA, 2016), with the potential to bioaccumulate and affect animal and human health (Osman et al., 2023).

Increasing numbers of studies demonstrate the occurrence of MPs in matrices to which humans are extensively exposed, including drinking-water (Gambino et al., 2022), food and beverages (Jin et al., 2019), with possible implications for human health associated with MPs and the release of chemical additives or other contaminants vectored by them (Gunaalan et al., 2020; Xia et al., 2022; Seewoo et al., 2023).

Cox et al. (2019) reported data on MP ingestion by American adults and children, which are estimated in the range of 81,000 to 123,000 MP per year, and in particular up to 4600 MP/year from shellfish, 5800 from tap water, beer and salt. Notably, annual intake of MPs from drinking water was calculated as 90,000 units if only bottled water is consumed and 4,000 if only tap water is consumed.

Also inhaled MPs may enter the digestive system via mucociliary

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clearing, but this route of exposure appears to be secondary to food and beverages. Thus, drinking water and food contamination substantially contribute to human exposure to MPs and contaminated food in particular seafood and processed food must be considered primary sources (Danopoulos et al., 2020; Milne et al., 2023).

In 2022, significant levels (i.e. 1.6 μg/mL) of plastic polymers were quantified for the first time in the whole blood of healthy humans, and polyethylene terephthalate, polyethylene (PE) and polystyrene (PS) were the most widely present (Leslie et al., 2022). There is increasing evidence that MPs occur in human tissues and fluids (Chen and Lin, 2024; Zuri et al., 2023) including lung, breast milk, testis tissue and semen, placenta (Ragusa et al., 2021) and the highest concentration was found in fecal samples of both adults (about 30 MP/g) and infants (about 25 MP/g). Therefore, microplastic pollution represents not only an environmental concern, but also a public health issue.

Although it is a shared opinion that the most significant route MPs enter the human body is via ingestion (Danopoulos et al., 2020), little is known about the fate and the interactions of MPs along the human digestive tract. Previous investigations have shown that after oral exposure MPs have a negative impact on gut microbiota in aquatic and terrestrial animals, including laboratory rodents, where intestinal dysbiosis, metabolism alteration and gut inflammation were promoted (Souza-Silva et al., 2022). Recent studies demonstrated that mice exposure to MP and NP induced hematopoietic toxicity via the crosstalk of gut microbiota, metabolites, and cytokines (Jing et al., 2022). As to humans, the impact of chronic exposure to MPs on the gut microbiome is still far from being understood. As recently suggested, the fastest way to resolve the scientific unknowns on human exposure is to implement research addressing knowledge gaps and toxicological effect mechanisms (Leslie and Depledge, 2020).

With the aim to investigate and comprehend the mechanistic key role of MP-microbes' interactions in the gut, we presently propose the use of *in vitro* intestinal models as a valid scientific approach that allow to study the perturbation that MPs cause in food digestion and microbiota metabolism and the dynamics from the oral cavity to the distal colon, before setting up a human interventional trial or animal research (Lu et al., 2019; Fournier et al., 2023). We also aimed to give a contribution to the *in vitro* evaluation of MP toxicity for humans and development of standardized protocols (Forest and Pourchez, 2023).

For instance, the toddler mucosal artificial colon was adopted to study the modifications of colon microbiota composition and metabolism (volatilome) induced by the exposure to polyethylene (PE) MPs (Fournier et al., 2023). In this model, PE MPs induced gut microbial shifts increasing the abundance of potentially harmful pathobionts, associated to a decrease in butyrate production and major changes in volatile organic compound synthesis. A further study, based on adult gastrointestinal model, evaluated the effects of partially digested bioplastics (i.e. polylactic acid) MPs on the human colonic microbiota composition and on short-chain fatty acids (SCFA) production, highlighting the alteration of microbial metabolism (Jiménez-Arroyo et al., 2023).

Since humans are never exposed to just one polymer, in this work we tested the simultaneous exposure to two polymers among the most present in the environment (Purayil et al., 2024), in foods and drinks (Yadav et al., 2022) and in human blood (Leslie et al., 2022), namely PE and polystyrene (PS). A static intestinal model based on INFOGEST digestion protocol (Brodkorb et al., 2019) followed by fermentation by human colon microbiota was employed using MICODE (Multi-Unit *in vitro* Colon Model) (Nissen et al., 2021). The perturbation of the microbiota and the colonic fermentation performance induced by two different concentrations of a mixture (1:1) of 1–4 μm diameter PE and PS MPs was investigated. Microbiota composition at the baseline was compared to those at different time points, applying microbiomics (qPCR) and an untargeted metabolomic approach based on volatile metabolites (SPME GC–MS) in a multivariate statistic showcase.

2. Material and methods

2.1. Microplastics

Polystyrene Microspheres (3 μm mean diameter) were purchased from Polysciences (Warrington, PA, USA). Polyethylene Microspheres (1–4 μm diameter) were purchased from Cospheric LLC, Somis, CA, USA. MPs were weighted and aliquoted in two working amounts, i.e. 0.166 g and 0.033 g and finally resuspended in sterile Milli-Q water (Millipore, Burlington, MA, USA). The working amounts chosen for this work were derived from the mass-based data reported by Senathirajah et al., (2021) corresponding to a maximum of 5 g of MPs ingested weekly per person, considering an average of 30 meals per week. The maximum dose of 0.166 g per meal was associated with a lower one corresponding to 1/5 of the maximum. These doses fall within the extensive range of ingestion estimation reported in the literature (De Boever et al., 2024).

2.2. Experimental workflow

Briefly, MPs were processed for oro-gastro-duodenal digestion (INFOGEST protocol) because digestive secretions may change physicochemical particle characteristics (Stock et al., 2020) and their contents of plasticizers, stabilizers, and flame retardants (Chen et al., 2021), thus modifying their effects on the colon microbiome. Digested MPs were centrifuged for 10 min to discard liquid, and vacuum dried. Recovered MPs were weighted and transferred in MICODE for proximal colonic fermentation, using human colon microbiota (HCM). The shifts of the principal populations of HCM and its metabolites that occurred with fermentation were then studied.

2.3. Human colon microbiota

HCM was obtained from fecal samples of three lean healthy individuals. The number and type of volunteers, and the following procedures were in accordance with previous protocols (Arnal et al., 2021; Nissen et al., 2023; Oba et al., 2020). Fecal donations were obtained from three healthy subjects, (either female and male) aged between 30 and 45 y. Donors did not undergo antibiotic treatment for at least 3 months prior to stool collection, did not intentionally consume pre- or probiotic supplements before the experiment, and had no history of bowel disorders. Additionally, the donors were not smokers, not chronically consuming any drug, and not alcoholic drink consumers. Fecal samples were donated two times (with an interval of seven days) for the two biological replicates. To collect feces, donors were provided and instructed to use a collection kit, which includes a stool collector (Sarstedt AG & Co. KG, Nümbrecht, Germany) and an anaerobic jar with a O_2 catalyst (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA). Fecal samples c15ollected by volunteers in a sterile container and placed in an anaerobic jar with oxygen catalyst were transferred to the laboratory and processed within 2 h. HCM was obtained by homogenizing 2 g of each donation in 54 mL of pre-reduced phosphate buffered saline (PBS) (Nissen et al., 2021; 2023). The donors were informed of the study's aims and procedures and gave their verbal consent to the use of the fecal material in the experiments, in agreement with the ethics procedures required by the Bioethics Committee of the University of Bologna.

2.4. In vitro digestion and fermentation

Gastroduodenal digestion of MPs was performed using the INFOGEST method as previously reported by Brodkorb and coauthors (Brodkorb et al., 2019). PE and PS were aseptically weighted, suspended in Milli-Q water (0.2 g/ml) and processed for *in vitro* digestion as previously described. Briefly, the MPs (PE and PS) were suspended in Milli-Q water (0,2 g/ml) and mixed at 1:1 with simulated salivary fluid (SSF, pH = 6,5) containing human salivary α-amylase (Merck, Missouri, USA)

and then incubated on an orbital shaker for 2 min at 37 °C. Then, simulated gastric fluid (SGF) containing porcine pepsin (Merck, Missouri, USA) was added, providing an enzymatic activity of 2000 U/mL in the final digestion mixture. The pH was adjusted to 3, and the samples were incubated for 120 min at 37 °C with continuous agitation. After the gastric stage, simulated intestinal fluid (SIF) containing bovine bile (10 mM of bile salts in the final mixture) and porcine pancreatin (Merck, Missouri, USA) was added to reach 100 U/mL of trypsin activity and the digested samples were set to pH 7 and incubated for additional 120 min. To stop the digestion process, the samples were refrigerated at 4 °C. After digestion, PE and PS MPs were washed three times with 10 ml of Milli-Q water, recovered by centrifugation, dried by vacuum, mixed 1:1 (by weight) and kept at −80 °C until use in MICODE.

Short-term batch proximal colon fermentations were conducted for 24 h in independent vessels using the *in vitro* colon model MICODE (Nissen et al., 2021; 2023). The preparation of the experiments was made according to published procedures (Wang et al., 2020) and was previously described in detail (Nissen et al., 2021; 2023). Briefly, fermentation vessels were filled aseptically with 90 mL of basal medium (BM) and the bioreactor headplates were mounted, including previously sterilized and calibrated sensors, i.e., pH and DO_2 (Dissolved Oxygen) sensors, and left running to reach and maintain the proximal colon ecological conditions (0.0 % of Dissolved Oxygen and pH 5.75). Anaerobic condition (0.0 – 0.1 % w/v of DO_2) in each bioreactor was obtained in about 30 min flushing with filtered O_2-free N_2 through the mounted-in sparger of Minibio reactors (Applikon Biotechnology BV, NL), and was constantly kept over the experiment. Temperature was set at 37 °C and stirring at 300 rpm, while pH was adjusted to 6.75 and kept throughout the experiment with the automatic addition of filtered NaOH or HCl (0.5 M) to mimic the conditions of the distal region of the human large intestine. Once the exact environmental setting was reached, the three vessels were aseptically injected with 10 mL of fecal slurry (10 % w/v of human feces to a final concentration of 1 %, w/v) and two of them supplemented with 0.166 g or 0.033 g of pooled digested PE and PS (1:1); the third vessel was set as blank control, named "water" (Milli-q water, basal medium and 1 % fecal slurry). Batch cultures were run under controlled conditions for a period of 48 h during which samples were collected at 3 time points (Baseline, 24 and 48 h). The baseline (BL) was defined on the first pH changes detected by Lucullus (1 read/10 s) via the bioreactors' pH sensors. For this work, the BL was set after 1.15 ± 0.09 h. Samplings were performed with a dedicated double syringe filtered system (Applikon Biotechnology BV, NL) connected to a float drawing from the bottom of the vessels without perturbing or interacting with the bioreactor's ecosystem. To guarantee a strict control of fermentation parameters the software Lucullus 3.1 (PIMS, Applikon Biotechnology BV, NL) was used which allowed to record the stability of all settings during the experiment. Fermentations were conducted in duplicate biological independent experiments, using for each a new pool of feces from the same three healthy donors. Effluates were collected for all the analytical assays as describe below.

2.5. Processing of MICODE effluates

Parallel and independent vessels for MP exposure were run for 48 h after the adaptation of the fecal inoculum, defined as the baseline (BL). The entire experiment was based on 3 conditions (water, mix 0.166 and mix 0.033) and 3 time points (BL, 24 and 48 h) in biological duplicate (n = 18). The three conditions were mix 0.166 = digested homogeneous mix PE and PS MPs in the load of 0.166 g; mix 0.033 = digested homogeneous mix of PE and PS MPs in the load of 0.033 g; and water = blank control). Samples of the different time points were used for microbiota characterization by absolute quantification with Real Time quantitative PCR of 16S rRNA or monocopy genes and for metabolite characterization by untargeted gas chromatography with SPME GC–MS (solid phase micro extraction gas chromatography mass spectrophotometry) approach. After sterile sampling of 5 mL of bioreactor contents, samples were centrifuged at 16,000 × g for 7 min to separate pellets and supernatants, which were used for bacterial DNA extraction and SPME-GC–MS analysis, respectively. The pellets were also washed twice in PBS prior DNA extraction. Microbial DNA extraction was conducted immediately after sampling to avoid reduction of *Firmicutes* content. DNA and SPME-GC–MS samples were stored at −80 °C prior analyses. Technical replicates of analyses were conducted in duplicate for SPME GC–MS (n = 36) and in triplicate for qPCR (n = 54).

2.6. Quantification of MPs

Samples of batch cultures at the 3 time points were collected and stored at −20 °C prior to pyrolysis–gas chromatography–mass spectrometry (Py-GC–MS) analysis of MPs. Each sample was quantitatively transferred into previously weighted Erlenmeyer flasks, dried at 90 °C and weighted. Then, dried samples added with 25 mL of distilled water and Fenton reagent (hydrogen peroxide 30 % v/vs and iron (II) sulphate in acetic/acetate buffer solution) were oxidised 2 h with 1 h in a ultrasonic bath (ElmaSonic S). The mixture was filtered on quartz fiber filter (Munktell, pore size 0.3 μm, diameter 25 mm, pretreated in a furnace at 600 °C for 4 h) and the filter dried in an oven at 90 °C. Portions of the filter were cut, weighed, transferred into pyrolysis cup, added with internal standard (5 μL 100 mg L^{-1} tri-*tert*-butylbenzene in toluene) and analysed by Py-GC–MS.

Py-GC–MS analyses were conducted at 600 °C using an EGA-PY 3030D pyrolyzer (Frontiel Lab) coupled with a 7890B- 5977B GC–MS (Agilent Technology) using a fused silica capillary column HP-5MS (30 m x 250 μm x 0.25 μm, stationary phase 5 %-diphenyl- 95 %-polysiloxane) working at 50 °C (2 min) ramped 7 °C min^{-1} to 310 °C (8 min). Mass spectra were recorded in the *m/z* range 30 – 600 under 70 eV electron ionisation with interval at 2.6 scan s^{-1}. Ion source and quadrupole temperature were set at 230 °C and 150 °C, respectively.

Polymer quantification was accomplished by the internal standard method with calibration curves constructed for PE (4.5–154 μg, n = 7 R^2 = 0.982 Microplastic calibration set, Frontier Lab pyrolysis markers C14-C20 *n*-alka-α,ω-dienes, quantitation ion *m/z* 82) and PS (0.5 – 194 μg, Frontier Lab dissolved in CH_2Cl_2, n = 11, R^2 = 0.981, pyrolysis marker styrene trimer, quantitation ion *m/z* 312).

Procedural blanks and blanks for air contamination were performed at each set of analysis; solvents were filtered at 0.3 μm. Inspection of the pyrograms of the blank control did not reveal the presence of pyrolysis markers of polypropylene, poly(ethylene terephthalate), polyamides, poly(methyl methacrylate). The precision as % RSD of the Fenton procedure evaluated on matrix-matching sewage sludge was < 25 % and < 33 % on replicate Py-GC–MS filter analyses. The limit of quantitation (LOQ) was 0.5 μg and 4.5 μg for PS and PE, respectively.

2.7. DNA extraction

DNA was extracted from 0.25 g/mL of MICODE effluates, washed twice in PBS, at each time points (BL, 24 h, and 48 h) using the NucleoSpin Stool DNA Extraction Kit (Macherey Naegel, Darmstadt, Germany), with little modifications, namely: 20 % higher concentration of lysis buffer, 50 % longer time on lysis incubation and horizontal shaking, 75 °C of lysis temperature. Nucleic acid purity was tested on BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK) and samples were selected for a purity level within the ranges 1.8–2.0 and 2.0–2.2 for λ260/λ280 and λ260/ λ230, respectively.

2.8. Absolute enumeration of bacterial groups by qPCR

Enumeration of bacterial groups was made by qPCR to evaluate changes in the microbiota after fermentation (Nissen et al., 2021; 2023; Tamargo et al., 2022) following previously reported protocols (Nissen et al., 2021; 2023). Fifteen different bacterial taxa (Table S1) were assessed by qPCR on a QuantStudio 5 System (Applied Biosystem,

Thermo Fisher, USA). Selection of bacterial taxa of the microbiota was scientifically based on ecological indicators of eubiosis and dysbiosis states of the HCM. The 15 taxa selected are those typically modulated by the different components of the food intake in a short-term response based on substrate fermentation. The total Eubacteria, the *Firmicutes* and the *Bacteroidetes* contents were determined to provide the total sum and the quantification of the two major phyla of the microbiota. *Bifidobacteriaceae*, *Bifidobacterium longum*, *Lactobacillales*, *Clostridium* group IV, *Faecalibacterium prausnitzii*, *Bacteroides – Prevotella* (BPP) group and *Akkermansia muciniphila* were chosen as beneficial bacteria. *Enterobacteriaceae*, *Escherichia coli*, *Desulfovibrio* spp., *Atopobium – Collinsella* (ATOP) group and *Clostridium* group I were chosen as opportunistic bacteria.

2.9. Volatile organic compounds (VOCs) by SPME GC–MS

VOCs were evaluated through an Agilent 7890A Gas Chromotograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent Technologies 5975 mass spectrometer operating in the electron impact mode (ionization voltage of 70 eV) equipped with a Chromopack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The Solid Phase Micro-Extraction (SPME) GC–MS procedure and the identification of volatile compounds were carried out according to previous reports, with a few minor modifications (Nissen et al., 2023). Mass spectra were cleaned using the BSB (background subtraction) function (ChemStation, Agilent Technologies, USA) to remove the spectrum related to a sample from a sample-free column in order to reduce contributions from the column.

Identification of molecules was carried out by searching mass spectra in the NIST 11 MSMS library (NIST, Gaithersburg, MD, USA). Each VOC was absolutely quantified in mg/kg (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg) (Nissen et al., 2021; 2023). Samples at 24 and 48 h were compared to the baseline and values were expressed as shifts. All results were expressed as normalized mean values obtained from technical duplicates from two independent experiments.

2.10. Data processing and statistical analysis

For microbiomics, each dataset relative to quantification of a single bacterial taxon was processed for MANOVA (Multivariate ANOVA) including two categorical predictors: “time” and “matrix”, then Duncan multiple range *post hoc* test was applied for both categories to evidence significant differences (Table S2). The shifts in abundance were calculated as $Log_2(F/C)$ (Hill et al., 2017), that represents the ratio of time-point/baseline expressed as Log_2 fold change. These results are presented as boxplots in R environment (Spitzer et al., 2014) and are supported with extensive statistics in Table S3. The *firmicutes/Bacteroidetes* ratio is provided in Table S4.

For metabolomics of organic acids, phenols and indole, one-way ANOVA ($p < 0.05$) was used to determine significant variables in the dataset, then Tukey’s *post hoc* test was applied for both categories to evidence significant differences (Table S5). The significant variables were picked and their values relative to intermediate time points and endpoints were subtracted from the values at the baseline of fermentation and then normalized with the mean centering method in a scale from 1 to −1 and plotted in a box and whiskers style. Absolute quantification of selected VOCs at the beginning of colonic fermentation is presented in Table S6. Significance of Spearman-rank correlation analysis is presented in Table S7. Each dataset was considered for Normality and Homoscedasticity before performing ANOVA/MANOVA models. Statistics and graphics were made with Statistica v.8.0 (Tibco, Palo Alto, CA, USA).

3. Results

3.1. MPs quantification

In the present study dried samples from colonic fermentation and procedural blanks were subjected to Fenton oxidation to reduce the organic matrix in accordance with other studies on similar matrices (Yan et al., 2022). MPs trapped onto the membrane filters were analysed by Py-GC–MS to confirm the presence of MPs and evaluate actual concentrations. In the resulting pyrograms of control samples, peaks associated to the organic matrix were clearly visible (essentially pyrolysis products of lipids, fatty acids), indicating that the organic matrix was not totally removed by the Fenton treatment. Pyrograms of treated samples exhibited the typical fingerprinting of PE (characterised by triplets of *n*-alka-α,ω-diene, *n*-alk-1-ene, *n*-alkane) and PS (characterised by styrene monomer, dimer and trimer) attesting the presence of the MPs throughout the experiment, as shown in Figure S1. The concentrations of PE and PS averaged during the fermentation experiment are reported in Table 1, while scanning electron microscope (SEM) images showing MPs into the MICODE vessels are shown in Figure S2. The difference of the measured PS:PE ratio from the nominal 1:1 value could be caused by differential segregation of particles, for instance adhesion to labware during analytical sample pretreatment (Kosuth et al., 2023), or possibly by PE partial degradation by human gut bacteria, as recently reported (Jang et al., 2024).

3.2. Quality controls for the validation of MICODE protocol

To validate the MICODE experimental approach in the version of fecal batch of the human distal colon, we chose to monitor and check some parameters as quality controls (QC) related to the fermentation mechanics, the HCM metabolomics and microbiomics at the end of fermentations and in comparison to the baseline. QCs of the fermentation mechanisms were relative to pH and DO (Dissolved Oxygen) and were monitored through the whole fermentation procedure (1 read/10 s) by MICODE integrated software Lucullus 3.1 (PIMS, Applikon Biotechnology BV, NL) via the pH sensors and DO sensors of MICODE (Applisense, Applikon Biotechnology, BV, NL) (Nissen et al., 2021; 2023).

Adopted QCs for microbiomics were relative to maintaining of cultivation through the whole colonic fermentation system of absolute quantitation of total bacterial load and of oxygen sensitive *Faecalibacterium prausnitzii*. The first QC is to monitor that no sudden loss of microbiota had occurred and that the HCM was kept quantitatively representative (Nissen et al., 2021; 2023). The second is to monitor that the oxygen gradient was kept unharmful for the microbiota (Huyer et al., 2021).

Adopted QCs for the metabolomic were some stool-related compounds, namely urea, 1-propanol, butylated hydroxy toluene, and indole, that were adsorbed at the same retention times spreading across the complete chromatogram and were similarly quantified for each GC/MS analysis. All indicators confirmed the validity of MICODE to study colon microbiota ecology up to 48 h.

Table 1
Concentration (mg/g dry weight) of PE and PS MPs in MICODE vessels (mean values ± SD, averaged over the duration of the experiment).

Sample	PS	PE	PS + PE
mix 0.166	3.8 ± 1.9	1.4 ± 0.7	5.2
mix 0.033	0.7 ± 0.5	0.5 ± 0.1	1.2
water	< LOQ	< LOQ	< LOQ

LOQ (Limit of Quantitation) was 0.5 μg and 4.5 μg for PS and PE, respectively.

3.3. Microbiota shifts induced by MP mix during colonic fermentation

Absolute enumeration and the shifts in abundance of 15 selected bacterial groups during colonic fermentation induced by digested MPs were obtained by qPCR (Fig. 1A-C and Table S3). The core microbiota of the human colon was quantified, assessing the quantity of total bacterial content and that of the two principal HCM phyla, i.e. *Firmicutes* and *Bacteroidetes* (Fig. 1A). No significant shifts were recorded for total *Eubacteria* loads after 48 h of colonic fermentation at the two MP doses. However, at the endpoint of fermentation the abundance of total *Eubacteria* in the bioreactor with the highest concentration of MPs was 2.36 times higher than the abundance in the blank control ($p < 0.05$). Considering *Firmicutes*, no significant shifts were observed at any time points and any conditions ($p > 0.05$). The *Firmicutes* abundances surged with both MP mix, but not in the blank control with water. The abundance scored by "mix 0.166" was significant and 2.23 times higher than that scored by the control ($p < 0.05$). Differently, the *Bacteroidetes* abundance was significantly reduced with major severity in the bioreactor with the highest concentration of MPs, although in a time- and sample- independent manner ($p > 0.05$).

A renowned ecological indicator of microbial activity related to possible state of eubiosis for the gut colon microbiota has been considered, namely the *Firmicutes* to *Bacteroidetes* ratio (F/B), obtained from values of qPCR absolute quantifications (Table S4). At the baseline of colonic fermentations, the ratio values confirmed the healthy conditions of the stool donors, as the values were all below the value limit of 2, that indicates the limit of eubiosis of HCM. After 24 h of fermentation the F/B surged in all bioreactors, although differences reached significance only for "mix 0.166" ($p > 0.05$). After 48 h, at the endpoint of the experiment, the F/B surged in a significant manner ($p < 0.05$) in both bioreactors containing the MP mix, confirming a detrimental impact of MPs on the ecology of human colon microbiota. The impact severity was concentration- and time- dependent ($p < 0.05$): after 48 h of exposure to "mix 0.166" the F/B was significantly higher (65.25 %) than that calculated at 24 h, and significantly higher (59.52 %) than that obtained after exposure to "mix 0.033" at the same time point.

Among the beneficial bacteria groups present in the HCM, seven taxa from different taxonomic levels have been selected and quantified (Fig. 1B). The shifts related to *Lactobacillales* showed a trend dependent on time and MP concentration (both $p < 0.05$). In fact, in the sample "mix 0.166" after 48 h of colonic fermentation the growth of *Lactobacillales* was 2.64 times higher than in the sample "mix 0.033" at the same sampling point.

The shifts related to *Bifidobacteriaceae*, a fundamental healthy family of the human colon microbiota, had a different trend, as only the highest concentration of the MP mix was able to significantly reduce its abundance ($p < 0.05$). This reduction was time-dependent, and in comparison to the control, the *Bifidobacteriaceae* presence in the sample "mix 0.166" was almost three times lower ($p < 0.05$). The described scenario was aligned with the family member *Bifidobacterium longum* which was significantly underrepresented after 48 h of exposure to the highest concentration of MPs ($p < 0.05$).

The major detrimental effect of MP mix on HCM beneficial population was observed for the *Clostridium* group IV, which was drastically reduced already after 24 h of exposure and almost completely depleted at the end point of colonic fermentation, when the *Clostridium* group IV accounted as 5.94E + 06 ± 3.65E + 06 cells/mL. A notable reduction was also observed for *Faecalibacterium prausnitzii*, that is considered as a next generation probiotic (Martin et al., 2017) capable of modulating the production of butanoic acid, a fundamental energy brick for the microbiota and the host colonocytes. Exposure to MPs had a mild effect on the BPP group (*Bacteroides – Prevotella – Porphyromonas*), that in the gut is mainly represented by *Bacteroides* and includes important dietary bacteria. A surge in this group is generally a good indicator of eubiosis, because it contributes to lowering the F/B ratio. The reduction of HCM beneficial bacteria after exposure to MPs was observed also on the probiotic *Akkermansia muciniphila* (Miranda et al., 2023), although not as severe as for other taxa.

Among the opportunistic bacterial groups present in the colon, five taxa (Fig. 1C) were selected and quantified, namely the family *Enterobacteriaceae*, the *Atopobium – Collinsella* (ATOP) group, the *Clostridium* group I, the genus *Desulfovibrio* spp., and the species *Escherichia coli*. The shifts recorded by *Enterobacteriaceae,* and *E. coli* showed a similar trend, showing overrepresentation in samples containing the highest MP concentration at any time point. As to *E. coli*, the increase induced by "mix 0.166" was severe, more than the double with respect to the control and accounting for 1.56E + 05 ± 1.04E + 05 cells/mL. *E. coli* is an opportunistic commensal, that can trigger the host to pathologies, depending on the virulence of the overgrowing strain.

The shifts relative to *Desulfovibrio* spp. indicated an increase of abundance after 48 h of colonic fermentation and exposure to MP mix, depending on fermentation time ($p < 0.05$), but not on MP concentration. As to the ATOP group, severe overrepresentations were recorded for both MP concentrations at any time points. In particular, the increases induced by MP mix were time- and concentration- dependent ($p < 0.05$), with the highest concentration of MPs achieving more than the double of what recorded at the lowest concentration, at the same time point.

Finally, the exposure to MPs also fostered the *Clostridium* group I, in a time- and concentration- dependent manner ($p < 0.05$), reaching a maximum value after 48 h of incubation at the highest MP concentration, with an increase 3.8 times higher than that recorded at the lowest MP concentration at the same time point.

3.4. MPs affect the metabolic profiles of beneficial and detrimental compounds during colonic fermentations

In order to evaluate the impact of MP mix on the metabolic performances of the colon microbiota, the production of some positive or negative bioactive microbial metabolites was assessed by an untargeted (SPME) GC- MS analytical approach. Specifically, the shifts in loads from the baseline to the intermediate (24 h) and the endpoint (48 h) of fermentations of 7 short and medium chain organic acids in the form of volatile organic compounds (VOCs) with reported beneficial activities on the health of the host, and 5 aromatic compounds with reported negative effects toward the host were processed as follows: i) each single compound was normalized, with the mean centering method, within its dataset, which included cases from "mix 0.166" and "mix 0.033", and the blank control at three different time points; ii) the baseline dataset (Table S6) was then subtracted to the intermediate and endpoint datasets; iii) *post-hoc* analysis was performed to compare the sample productions of a single molecule (Tukey's test, $p < 0.05$). The plots of organic acids (Fig. 2) show the differences recorded between the reactors containing the MP mix and the bioreactors without MP added (BL). In particular, all the organic acids were significantly reduced after 48 h of colonic fermentation inside the bioreactor with "mix 0.166" ($p < 0.05$), and 4 of them were under limit of detection (LOD); inside the bioreactor with "mix 0.033" 3 out of 7 organic acids were not different from the BL ($p > 0.05$) and 4, namely acetic, hexanoic, octanoic and nonanoic acid showed lower levels than in BL ($p < 0.05$).

The plots of aromatic compounds show a clear trend (Fig. 3). In details, "mix 0.166" was the only one that determined a prominent production of all aromatic compounds, recording an increase that ranged approximately from 4 to 7 times higher than the BL. At the lower dose, "mix 0.033", MPs were still able to increase the production of VOCs, but in a milder way, doubling the p-cresol quantity with respect to BL.

3.5. Correlations between changes of colonic microbiota and metabolites after exposure to MPs

Spearman Rank Correlations ($p < 0.05$), two-joining-way heatmaps,

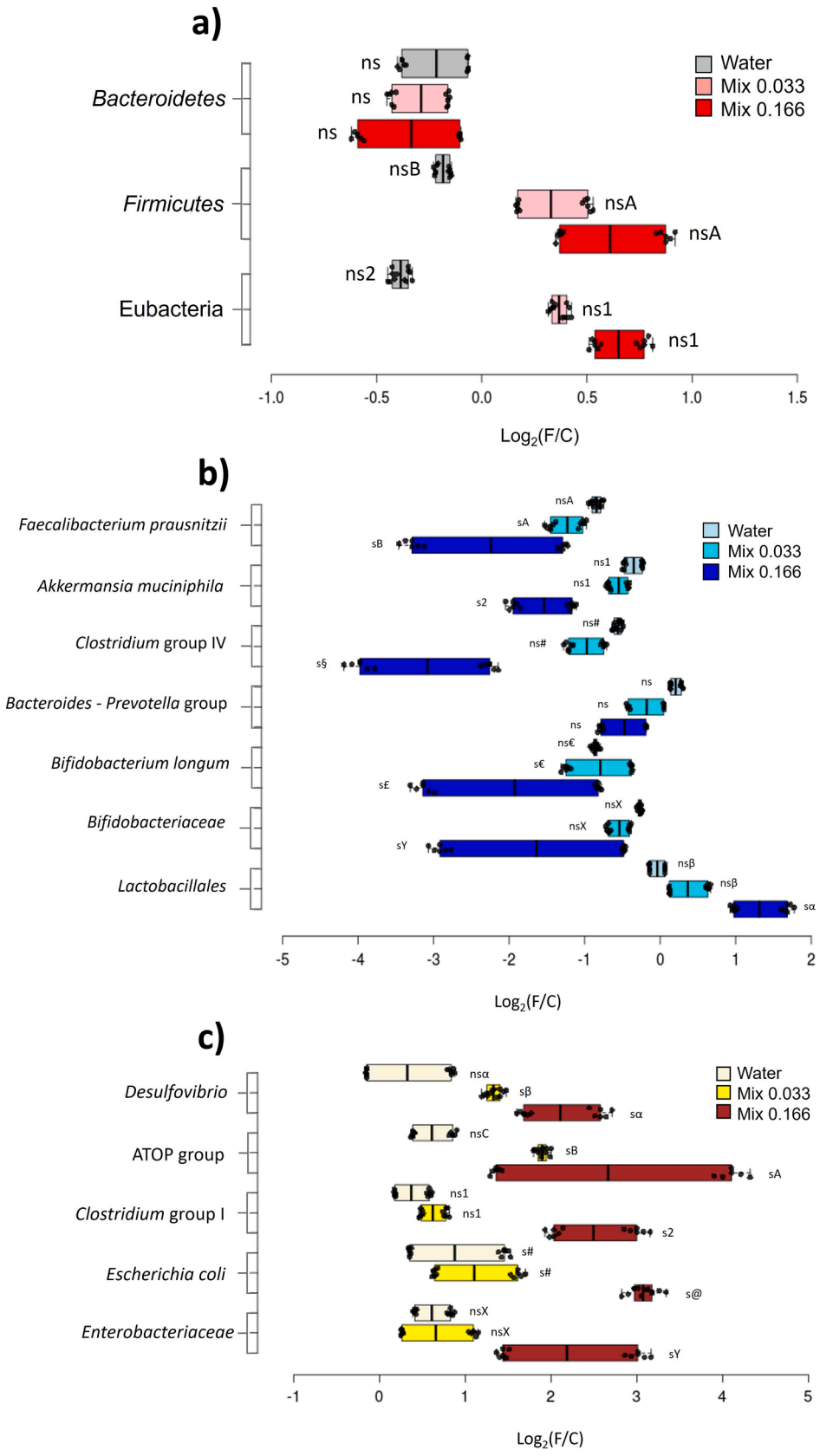

Fig. 1. Changes in respect to the baseline of fermentation expressed as $Log_2(F/C)$. Changes of core-microbiota taxa measured by qPCR during colonic fermentation, including values of 24 h and 48 h timepoints. Baseline values of absolute abundances and values of changes are reported in Table S3. a) Total eubacteria, *Firmicutes* and *Bacteroidetes*; b) Beneficial bacterial taxa; c) Opportunistic bacterial taxa. Letters and symbols indicate statistical differences as reported in Table S3. ns = not significant and s = significant are for the categorical predictor "fermentation time" and letters and symbols indicate significant differences among the three bioreactors for the categorical predictor "fermentation matrix".

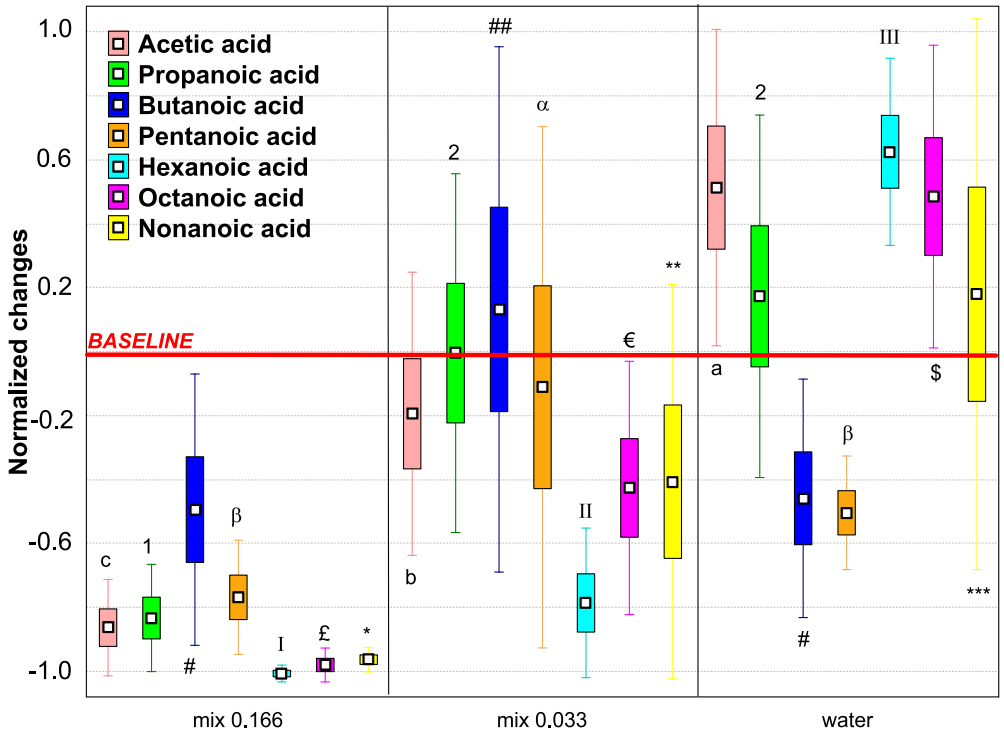

Fig. 2. Changes in the abundance of beneficial microbial VOCs metabolites, expressed as normalized scale from relative abundances with respect to the baseline (red line). The baseline absolute quantifications in mg/kg are reported in Table S6. Changes were recorded after 24 and 48 h of *in vitro* colonic batch fermentations with 0.166 g and 0.033 g of MP mix and a water control. Each plot is made with the raw data obtained from each time point and replicates. Samples were analyzed in technical duplicate from two independent experiments ($n = 4$). Marker = mean; box = mean ± S.D.; whiskers = confidence interval 0.95 %. Mix 0.166 = digested homogeneous mix of PE and polystyrene microplastics at the concentration of 0.166 g; mix 0.033 = digested homogeneous mix of polyethylene and polystyrene microplastics at the concentration of 0.033 g; water = blank control. Acetic acid: $p = 0.000005$; propanoic acid: $p = 0.003237$; butanoic acid: $p = 0.008444$; pentanoic acid: $p = 0.041515$; hexanoic acid: $p = 0.000001$; octanoic acid: $p = 0.000012$; nonanoic acid: $p = 0.013991$, by ANOVA ($p < 0.05$). Cases with different letters or numbers or symbols among a single independent variable are significantly different according to *post hoc* Tukey's test ($p < 0.05$).

and Pearson cluster analysis were performed by the comparison of two different normalized datasets, each derived from values of relative quantification (qPCR targets and VOCs) (Fig. 4). Significance of correlations is reported in Table S7. From the Pearson dendrograms, four main clusters were identified.

The first cluster (A1) related to bacterial taxa included BPP group, *Clostridium* group IV and the two bifidobacterial targets that have positive correlations with beneficial pentanoic, propanoic, acetic and hexanoic acids. In particular, in this cluster, strong positive correlations were observed for acetic acid to *Clostridium* group IV, *B. longum* and *Bifidobacteriaceae* (correlation strength = 0.83, 0.80, 0.80, respectively). In A2 cluster, the same bacterial variables were negatively correlated to nonanoic, octanoic and butanoic acids and to the detrimental aromatic compounds. The third cluster (B1) included *Enterobacteriaceae* and *E. coli*, *Clostridium* group I, *Lactobacillales* and ATOP group that showed negative correlations with beneficial pentanoic, propanoic and acetic acid. Lastly, the fourth cluster (B2) was related to the latter list of bacteria and mainly aromatic compounds as phenols and indole. In particular, strong positive significant correlations in this cluster were seen for p-cresol, indole and phenol, p-*tert*-butyl with respect to ATOP group (correlation strength = 0.72, 0.73, 0.89, respectively), and P24B with *Enterobacteriaceae* and *E. coli* (correlation strength = 0.80 and 0.77, respectively). The correlations found give robustness to the result datasets, indicating clear causalities for the interaction of microbes and their metabolites in the human colon microbiota exposed to MP mix.

4. Discussion

4.1. Microbiomics

Among the 15 selected groups analyzed, different changes in beneficial and opportunistic populations of the colonic microbiota were observed after exposure to MPs mix. As a general trend, the beneficial taxa tested (with the exception of *Lactobacillales*) suffered from the presence of MPs, depending on time of colonic fermentation and on concentration of MPs. The longer the time of fermentation and the higher the concentration of MPs, the more severe was the negative modulation. The observed overrepresentation of Eubacteria induced by the highest concentration of MPs is in agreement with the findings obtained with adult (Fournier et al., 2023) and infant (Fournier et al., 2023) by using *in vitro* gut models. However, both were in contrast with results obtained *in vivo* in mice (Jin et al., 2019; Lu et al., 2018). In our model, after 48 h of exposure to PE and PS MP mix, the *Firmicutes* abundance surged oppositely to that of *Bacteroidetes*, that is the reason why we obtained a significant increase of the F/B ratio. These changes could be explained hypothesizing that many residents taxa of the *Firmicutes* phylum had some competitive advantage by the presence of MPs, i.e. they could benefit of possible aggregation on plastic items (Béchon and Ghigo, 2022), while the *Bacteroidetes* are poorly provided of such abilities. Using the *in vitro* fermentation model, based on human feces, *Firmicutes* had shown to be augmented and *Bacteroidetes* to be reduced after PET microplastic exposure (Tamargo et al., 2022) in accordance with our observations. However, *in vivo* experiments on mice showed that *Firmicutes* were reduced after exposure to polystyrene MPs (Lu et al., 2018). It is generally accepted that the F/B ratio indicates a balance of the two major phyla of the microbiota. When this ratio is

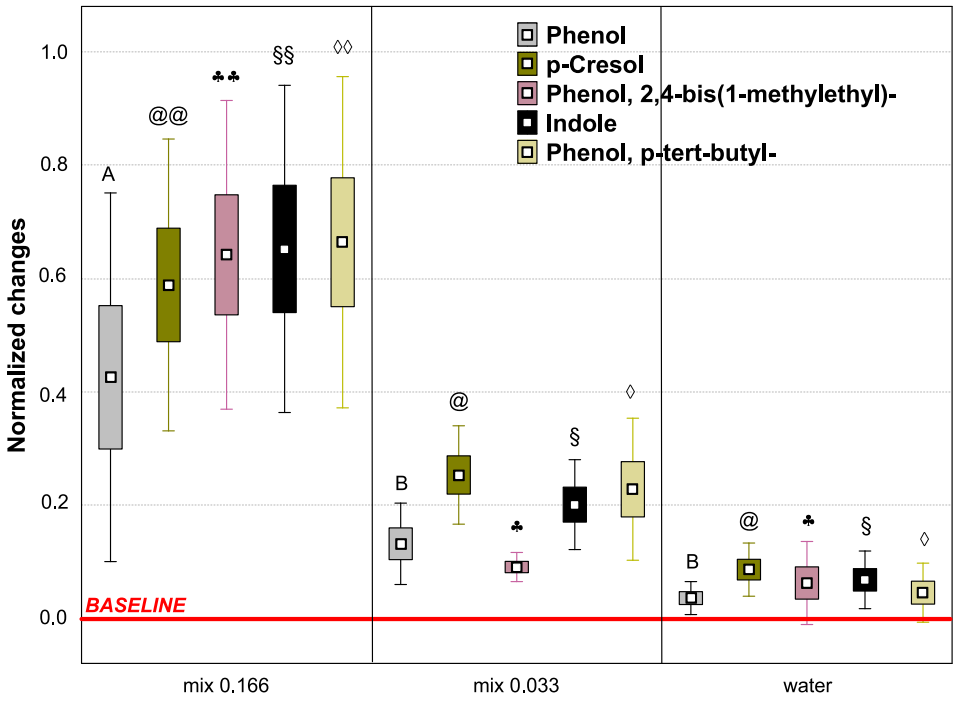

Fig. 3. Changes in the abundance of detrimental microbial VOCs metabolites, expressed as normalized scale from relative abundances with respect to the baseline (red line). The baseline absolute quantifications in mg/kg are reported in Table S6. Changes were recorded after 24 and 48 h of *in vitro* colonic batch fermentations with 0.166 g and 0.033 g of MP mix and a water control. Each plot is made with the raw data obtained from each time point and replicate. Samples were analyzed in technical duplicate from two independent experiments ($n = 4$). Marker = mean; box = mean ± S.D.; whiskers = confidence interval 0.95 %. Mix 0.166 = digested homogeneous mix of polyethylene and polystyrene microplastics at the concentration of 0.166 g; mix 0.033 = digested homogeneous mix of polyethylene and polystyrene microplastics at the concentration of 0.033 g; water = blank control. Phenol: $p = 0.006100$; p-cresol: $p = 0.000100$; phenol, 2,4-bis(1-methylethyl)-: $p = 0.000010$; indole: $p = 0.000060$; phenol, p-*tert*-butyl-: $p = 0.000003$, by ANOVA ($p < 0.05$). Cases with different letters or numbers or symbols among a single independent variable are significantly different according to *post hoc* Tukey's test ($p < 0.05$).

lower than 2, it indicates eubiosis of microbiota and a general host's well-being. Oppositely, a F/B ratio greater than 2 indicates dysbiosis, and inflammation, and it has been associated with several pathologies (Amabebe et al., 2021). Our results support the view that a high concentration of PE and PS into the gut tend to shift the human microbiota ecology towards a state of dysbiosis.

Exposure to MP mix caused underrepresentation of most of the beneficial microbial targets assessed, with the exception of *Lactobacillales*, which were increased by the presence of MPs. In a previous work *in vivo* conducted on mice, similar results were observed after exposure to polyethylene MPs (Li et al., 2020a). This class is mainly represented by *Enterococcaceae* and marginally by *Lactobacillaceae*, which are important mutualism actors within the host, and could be adjuvated by the presence of MPs, in particularly as a non-metabolic substrate that bacteria exploit to extend the colonies dimensions. In fact, enterococci are known to aggregate (Rodríguez-Melcón et al., 2018), and lactobacilli possess abilities of auto-aggregation (Zawistowska-Rojek et al., 2022), adhesion (Monteagudo-Mera et al., 2019), and interaction with polytetrafluoroethylene (Ibarreche et al., 2014). Exposure to MPs had a mild effect on the BPP group (*Bacteroides – Prevotella* group). Generally, this group was slightly reduced at the end point (48 h) only after exposure to the highest concentration of MP mix, as observed also in other *in vitro* intestinal model (Tamargo et al., 2022). *Clostridium* group IV and its resident member *Faecalibacterium prausnitzii*, regulators of the butanoic acid production within the host, were profoundly affected by the exposure to the MP mix. Similar findings were documented by previous *in vitro* studies, with PE MPs and infant gut microbiota (Fournier et al., 2023) and with PET MPs (Tamargo et al., 2022). Lastly, a further second-generation probiotic, namely *Akkermansia muciniphila*, was reduced by MP mix exposure but with less severity than other beneficial taxa. This evidence was also obtained in other studies, testing PE MPs both *in vivo* on mice (Li et al., 2020b), and *in vitro* using an intestinal model (Fournier et al., 2023).

About the opportunistic populations of the microbiota, a general increase in their abundance was observed after exposure to MPs mix, in a time- and concentration- dependent manner. For example, *Enterobacteriaceae* and *E. coli* were greatly fostered by MP mix. Similar results were previously reported for *E. coli* after exposure of adult human gut microbiota to PE MPs in *in vitro* intestinal models (Fournier et al., 2023; 30 Tamargo et al., 2022) and also for the whole Enterobacteriaceae family using infant gut *in vitro* model (Fournier et al., 2023). The growth competitive advantage of the members of this family, and in particular *E. coli,* in the presence of MPs could be assisted by the intrinsic abilities of these microbes to adhere on and colonize lipophilic surfaces (Rosato et al., 2022). In facts, many members of Enterobacteriaceae and in particular *E. coli* are able to interact with polystyrene surface (Rodríguez-Melcón et al., 2018) and polyethylene terephthalate (Yi et al., 2021). We also recorded an increase in *Desulfovibrio* spp., a genus of sulphate reducer that ferment proteins and generate compounds detrimental for the host health, such as thiourea, dimethyl and trimethyl sulfides, thiazoles, indoles and phenols (Hagiya et al., 2018).

Our findings at the genus level are new, but in line with two previous studies targeting higher taxonomic levels. In fact, the *in vitro* gut exposure to PE MPs generated results of overrepresentation of *Desulfobacterota* phylum (Tamargo et al., 2022) and *Dethiosulfovibrionaceae* family (Fournier et al., 2023; 2023].

In MICODE, MPs mix were also found to increase in a time- and dose-dependent manner the ATOP group, that is a key player in the onset of dysbiosis in humans. *Collinsella aerofaciens* is an ATOP member that is considered a marker of irritable bowel syndrome (IBS) linked dysbiosis (Gargari et al., 2024). This finding is new, since this group of bacteria was not considered in previous studies; moreover, it indicates MPs are

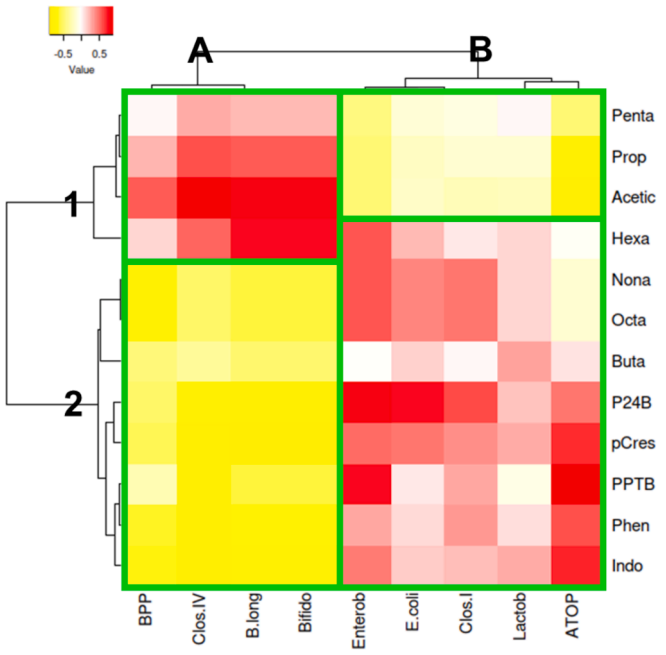

Fig. 4. Spearman Rank correlation analysis in a two-way joining heatmaps with Pearson dendrograms. Interomic correlations and clusterization of variables from the microbiomics and the metabolomics datasets. Significance of correlations is reported in Table S5. Penta = Pentanoic acid: Prop = Propanoic acid; Acetic = Acetic acid; Hexa = Hexanoic acid; Nona = Nonanoic acid; Octa = Octanoic acid; Buta = Butanoic acid; P24B = Phenol, 2,4-bis(1,1-dimethylethyl)-; pCres = p-Cresol; PPTB = Phenol, p-*tert*-butyl; Phen = Phenol; Indo = Indole; BPP = *Bacteroides – Prevotella – Porphyromonas* group; Clos.IV = *Clostridium* group IV; B.long = *Bifidobacterium longum*; Bifido = *Bifidobacteriaceae*; Enterob = *Enterobacteriaceae*; E.coli = *Escherichia coli*; Clos.I = *Clostridium* group I; Lactob = *Lactobacillales*; ATOP = *Atopobium – Collinsella* group.

environmental pollutants potentially capable to affect the functional intestinal diseases worsening their outcomes. The present work also shed some lights on the effect of MPs on the modulation of *Clostridium* group I, for which a time- and dose- dependent increase was recorded. To our knowledge, these is the first finding addressing the *Clostridium* group I modulation after exposure to MPs. Nevertheless, this result is not surprising since this group of bacteria is able to positively interact to abiotic surfaces (Rodríguez-Melcón et al., 2018) by using pili.

4.2. Metabolomics

Altogether, we observed that the beneficial compounds were poorly recovered from the effluates of the bioreactors in the presence of MP mix during colonic fermentation, indicating that the deputed beneficial part of the microbiota in those conditions was not able to produce them. In fact, a class of important bioactive beneficial compounds represented by organic acids from C2 to C9 was reduced depending on time of exposure and concentration of MPs. For example, the effect of the highest concentration of MPs on the reduction of butanoic acid could be related to the depletion generated by MP mix on the population of *Clostridium* group IV, a well-known butyrate-producer (Andersen et al., 2017). On the other hand, production of detrimental aromatic compounds was more severe when the microbiota was exposed to the MP mix. A class of aromatic compounds, as phenols and indole renowned for their detrimental effect on the host mucosa (Rahman et al., 2021; Ye et al., 2022), and mainly synthesized during the fermentation of proteins, were over produced when the microbiota was exposed to the PE and PS MP mix, depending on time and concentration. The augmented production of the detrimental *p*-cresol could be due to the increased growth of the *Enterobacteriaceae* family, the *Clostridium* group I and the ATOP group. In fact, all these taxa produce phenols and indoles in their toxic forms as metabolites of their amino acid fermentation, in particular tyrosine and tryptophan (Amaretti et al., 2019). In terms of correlations between microbiota shift and volatilome, we found an inverse correlation between butanoic acid synthesis and *Clostridium* group IV, and a significant correlation between *Enterobacteriaceae* family, the *Clostridium* group I and the ATOP group with different phenolic compound synthesis, including p-cresol. These correlations strengthen our experimental results.

The data here reported on metabolomics are new; due to the lack of research articles investigating this issue it is difficult to make a comparison with previous evidence. The focus on metabolomics represents an important strength of this study, together with the quantitative analysis of the content of MPs into the vessel medium. In facts, at the best of our knowledge, quantitative monitoring of the content of MP in the fermentation media was not performed in previous studies in which SEM, Raman and FTIR techniques were utilized for the quantitative determination of MPs in human colon and feces (Fournier et al., 2023; 2023; Ibrahim et al., 2020; Schwabl et al., 2019). Py-GC–MS is a thermoanalytical technique capable to inform on the mass-based concentration of MPs, a quantitative unit well fitted for risk assessment and data comparison. Further improvements in experimental exposure investigations should take into account quality criteria, including positive controls, that are under development for monitoring studies.

The limitations of this study may be due to the amount of MPs we used. Although it derives from studies that assessed reasonable quantities of MPs ingested (Senathirajah et al., 2021; De Boever et al., 2024) these values represent maximum estimates that are probably not found in the population of Western countries. In fact, in the healthy donors we enrolled, the doses of PS and PE measured were much lower. Furthermore, the estimated total amount of MPs in the human colon depends on many types of plastics and not only on PE and PS which although represent those most present in the food chain. Nonetheless, our study represents a proof of concept that the presence of these plastics in the gut can profoundly alter the microbiome. Nor can it be ruled out that in developing countries where contamination of foods and environment by MPs is much higher, these dangerous doses have not already been reached by the population.

5. Conclusion

The impact of high exposure rates of PE and PS on the colon ecology of human microbiota is significant, and includes the induction of opportunistic bacteria overgrowth, raising the numbers of *Enterobacteriaceae* and *E. coli*, *Desulfovibrio* spp., *Clostridium* group I and *Atopobium – Collinsella* group, and a contextual reduction on abundances of all the beneficial taxa analyzed, with the sole exception of *Lactobacillales.* We may hypothesize that the reduction in abundance of the beneficial taxa is not directly caused by the MPs, but rather by a higher competitive advantage offered to the opportunistic bacterial populations, able to exploit the presence of MPs as an inert surface to facilitate their adaptation to the colon ecosystem. We are aware that the concentration of PE and PS used do not reflect realistic average levels of ingested microplastics in humans, at least in Western countries. Despite this, considering the continuous increase in the presence of MPs in the environment and its heterogeneous geographical distribution, it cannot be ruled out that human exposure levels similar to those we tested have already been reached, or will be reached in a very near future.

For the first time metabolomics was adopted to study *in vitro* MP interaction with gut microbiota in a simulated colon ecosystem. Interestingly, the scenario is aligned to that of microbiota and confirmed by Spearman analysis which correlates the two “omic” approaches. Again, one may speculate that the mechanism behind this phenomenon is a different cell-surface interactions of the bacteria taxa with PE and PS MPs, resulting in a competitive advantage of their physiology in response to the altered ecological conditions. The metabolomic

approach highlighted that the microbiota shifts are consistent to its metabolic activity, which also makes realistic the hypothesis of an active interactions of colon population with MP and/or related molecules released in the gut milieu.

The results from the *in vitro* model provide a robust foundation for clinical applications and/or toxicological studies on microplastics as pollutants for humans, reporting quantitative results with higher translatability than those based on qualitative MP quantification or animal models. MICODE has proven to be a robust and versatile *in vitro* model and a suitable approach to describe the effects generated by food contaminants on microbiome and human health.

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7. Institutional review board statement

The study was conducted according to the guidelines of the ethics procedures required at the University of Bologna.

CRediT authorship contribution statement

Lorenzo Nissen: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Enzo Spisni:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Renato Spigarelli:** Methodology. **Flavia Casciano:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Maria Chiara Valerii:** Methodology. **Elena Fabbri:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Daniele Fabbri:** Writing – original draft, Resources, Methodology, Funding acquisition. **Hira Zulfiqar:** Investigation. **Irene Coralli:** Validation, Methodology. **Andrea Gianotti:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2024.108884.

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