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Perturbation of gut microbiota plays an important role in micro/nanoplastics-induced gut barrier dysfunction
Perturbation of gut microbiota plays an important role in micro/nanoplastics-induced gut barrier dysfunction†

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The widespread occurrence of microplastics (MPLs) and nanoplastics (NPLs), collectively abbreviated as M/NPLs, has markedly affected the ecosystem and has become a global threat to human health. Multiple investigations have shown that the chronic ingestion of M/NPLs negatively affects gut barrier function but the mechanism remains unclear. Herein, this research has investigated the toxic effects of pristine polystyrene (PS) M/NPLs, negatively charged carboxylated polystyrene M/NPLs (PS-COOH) and positively charged aminated polystyrene M/NPLs (PS-NH₂) of two sizes (70 nm and 5 μm in diameter) in mice. Gavage of these PS M/NPLs for 28 days caused obvious injuries to the gut tract, leading to the decreased expression of tight junction proteins. The toxicity of the M/NPLs was ranked as PS-NH₂ > PS-COOH > pristine PS. Oral administration of these M/NPLs resulted in marked gut microbiota dysbiosis. The M/NPLs-enriched genera generally contained opportunistic pathogens which are accompanied by a deteriorated intestinal barrier function, while most M/NPLs-decreased bacteria were beneficial microbes with known tight junction-promoting functions, implicating an important indirect toxic effect of gut microbiota dysbiosis in M/NPLs-induced gut barrier dysfunction. In conclusion, this research highlights the importance of gut microbiota in the toxicity of M/NPLs exposure on gut barrier function, providing novel insights into the adverse effects of M/NPLs exposure on human health.

1 Introduction

Plastic litter is ubiquitous throughout marine and terrestrial ecosystems and has led to significant environmental pollution.1 Although plastic is considered to be a long-lasting and stable material in the environment, it can break into microplastics (MPLs) and nanoplastics (NPLs), abbreviated as M/NPLs, through UV-radiation, mechanical abrasion and biologic degradation.2–5 In the different forming processes of M/NPLs, the surfaces of the microplastics will form various groups in the environment, giving new properties to this plastic debris. For example, the aging process caused by UV radiation could increase the negative charge on the surface of polystyrene (PS) MPLs.6 M/NPLs are also produced in industry as byproducts in manufacture16–19 or contaminants from plastic packaging.20,21 These ingested M/NPLs may have an inconceivable impact on human health. In 2018, a study from United European Gastroenterology (UEG) showed that MPLs exist in human faeces for the first time,22 warning of the potential risk of MPLs-ingesting organisms,13–15 byproducts in manufacture16–19 or contaminants from plastic packaging.20,21

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The intestinal barrier is essential for the maintenance of intestinal homeostasis and metabolism.22 Injury to the intestinal barrier integrity is closely associated with various diseases.24,25 Tight junctions (TJs) are intercellular adhesion complexes that are necessary for the barrier function of the

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†Electronic supplementary information (ESI) available: Material characteristics, animal testing data, cell experiment results and gut microbiota data. See DOI: 10.1039/d1nr00038a
intestinal epithelia and they are composed of transmembrane proteins, including claudins, occluding proteins, β-catenin and so on. The perturbation of TJ protein expression or disruption of TJ integrity is associated with various diseases, including inflammatory disease, diabetes and cancers. The gut microbiota plays a vital role in maintaining the barrier function of the intestinal epithelia. Increasing evidence shows that the gut microbiota is very sensitive to drugs, diet and environmental pollutants. It has been shown that environmental pollutants can alter the biological composition and diversity of intestinal microbes in living organisms, leading to energy metabolism disorders, inadequate nutrient absorption, decreased immune system function, or other symptoms of poisoning. Therefore, it is urgent to study the effect of cumulative exposure of M/NPLs on the GI tract and gut microbiota.

PS is not only a kind of plastic with high production but it is also one of the primary components of plastic debris observed in the environment. PS M/NPLs in environments are largely derived from the breakdown of plastic debris and are often transformed into different functionalized particles such as amino- and carboxyl-modulated forms. Although the harmful effects of PS M/NPLs on mammalian health have been intensively studied, the precise impacts of different chemically modulated PS M/NPLs remain largely unknown.

In this study, we selected three functionalized PS M/NPLs (5 μm and NPLs (70 nm), namely, pristine PS, PS-COOH, and PS-NH₂, to investigate the different influences of different PS modifications on the GI tract and gut microbiota. We also explored the potential mechanism underlying the dangers of PS M/NPLs to the gut tract, which highlighted the importance of gut dysbiosis in the harmful impact of M/NPLs on gut barrier function.

2 Materials and methods

2.1 Materials

Pristine PS M/NPLs, carboxyl-modified (PS-COOH) and amine-modified (PS-NH₂) PS M/NPLs (70 nm, 5 μm in diameter) were purchased from the BaseLine ChromTech Research Centre (Tianjin, China).

2.2 Characterization of the PS M/NPLs

The morphology and size of the M/NPLs were characterized by transmission electron microscopy (TEM, Tecnai G2 20 S-TWIN, Holland) at 200 kV. The sizes of the M/NPLs were statistically analysed from the TEM results by Nano measure. The aggregation and Zeta potentials in deionized water with a pH value of 7.0 were evaluated by a Zetasizer Nano ZS (Malvern Instrument Ltd, UK).

2.3 Animals and experimental design

C57/B6 mice (male, 8 weeks old, 24 g) were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and kept in a humidity-controlled room on a 12 h/12 h light–dark cycle with food and water available and acclimatized for 5 days. All of the animal experiments were performed in compliance with the National Institutes of Health regulations for the care and use of animals by the Chinese Society of Toxicology and Ethics Committee. The animals were divided randomly into 13 groups (6 animals per group): (a) control group, (b1) 2 mg kg⁻¹ nano PS-treated group, (b2) 0.2 mg kg⁻¹ nano PS-treated group, (c1) 2 mg kg⁻¹ micro PS-treated group, (c2) 0.2 mg kg⁻¹ micro PS-treated group, (d1) 2 mg kg⁻¹ nano PS-COOH-treated group, (d2) 0.2 mg kg⁻¹ nano PS-COOH-treated group, (e1) 2 mg kg⁻¹ micro PS-COOH-treated group, (e2) 0.2 mg kg⁻¹ micro PS-COOH–treated group, (f1) 2 mg kg⁻¹ nano PS-NH₂–treated group, (f2) 0.2 mg kg⁻¹ nano PS-NH₂–treated group, (g1) 2 mg kg⁻¹ micro PS-NH₂–treated group and (g2) 0.2 mg kg⁻¹ micro PS-NH₂–treated group. The treated groups were gavaged with different types of PS M/NPLs according to the weight of the mice (100 μL per 10 g body weight, suspended in distilled water) for 28 days, while the control group (NC) was given an equal volume of distilled water. Fresh faeces were collected from each mouse at 2 h after the last gavage, snap-frozen in liquid nitrogen, and then stored at −80 °C for subsequent analysis.

2.4 Blood biochemical analysis

Blood samples were collected in BD Vacutainer spray-coated K2EDTA tubes for whole serum biochemistry determinations. The mouse supernatant serum was collected by centrifugation at 3500 rpm for 20 min and the lower plasma was removed. Serum biochemistry markers, including alanine aminotransferase, aspartate aminotransferase, total bilirubin, creatine kinase, alkaline phosphatase, r-glutamine transferase and serum creatinine, were assayed by an automatic chemistry analyser (Celltac, MEK-6358; Nihon Kohden Co., Tokyo, Japan).

2.5 Histological analysis

For histological observation, tissues from the main organs of the heart, liver, spleen, lung, kidney, stomach, small intestine (duodenum, jejunum) and large intestine (colon) were taken from each animal and fixed in 4% buffered neutral formalin for 24 h, and the samples were embedded in paraffin and cut into 4 μm sections. The sections were stained with haematoxylin and eosin and imaged with an EVOS x1 core microscope (AMG, Life technologies). At least 20 slides from each group were analyzed.

2.6 16S rDNA pyrosequencing

The faecal material was snap-frozen in liquid nitrogen and stored at −80 °C. Faecal DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Polymerase chain reaction (PCR) amplification of the V3–V4 region of bacterial 16s rDNA was performed using universal primers (338F 5′-ACTCTTCGTTGAGGCAGCAG-3′, 806R 5′-GGACTACHVGGGTWTCTTAAT-3′) incorporating the FLX
Titanium adaptors and a barcode sequence. The PCR amplification and sequencing were carried out according to the recommendations of 454 Life Sciences, on a Roche Genome Sequencer GS FLX Titanium platform from the Majorbio Bio-Pharm Technology Co., Ltd (Shanghai, China).

2.7 Bioinformatic analysis
Bioinformatic analysis was performed on the Cloud Platform System from the Majorbio Bio-Pharm Technology Co., Ltd (Shanghai, China). Briefly, the raw sequence was trimmed according to the following rules: (1) each pyrosequencing read contained fewer than three mismatches in both the forward and reverse primers, (2) each pyrosequencing read had no ambiguous reads and less than 10 homologous reads, (3) chimeric sequences were excluded, and (4) all sequences were more than 200 bp in length. The trimmed sequences were clustered to OTUs defined by 97% similarity using the UPARSE method. The taxonomic assignment of the OTUs was performed by alignment with the Bacterial SILVA database (SILVA version 115; http://www.arb-silva.de/documentation/release-115). OTUs with more than 97% similarity were used for diversity (Shannon), richness (Chao), and rarefaction curve analyses using Mothur (version 1.30.1). Principal coordinate analysis, partial least squares discrimination analysis and average clustering based on Bray–Curtis distance were performed for each sample. The COG functions of the gut bacteria were predicted by PICRUSt software, which is incorporated in the Cloud Platform System of the Majorbio Bio-Pharm Technology Co., Ltd.

Cell viability test. Caco-2 cells were purchased from the Chinese Academy of Sciences Cell Bank. Caco-2 were grown in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (BI), 1% nonessential amino acids ( Gibco) and 1% penicillin–streptomycin. Cells were grown in an atmosphere of 5% CO2 at 37 °C. Cell viability was determined with the CCK-8 assay kits (Dojindo, Japan). Caco-2 cells were seeded in 96-well plates (Corning, USA) at a concentration of 8 × 104 cells per well. After incubation with different concentrations of PS M/NPLs (20 μg mL−1, 50 μg mL−1, 100 μg mL−1, 200 μg mL−1) for 24 h at 37 °C, the reagents of CCK-8 were added according to the manufacturer’s protocol, and absorbance was recorded at 450 nm by a microplate spectrophotometer (Tecan, Durham, USA). The mean absorbance of the nonexposed cells was taken as the reference value (100% cellular viability).

2.8 Cellular live/dead assay
Caco-2 cells were plated onto 96-well plates at a concentration of 2 × 104 cells per well and cultured for 7 days to form an integrated monolayer, then treated with 100 or 200 μg mL−1 PS M/NPLs for 24 h. After washing with phosphate-buffered saline (PBS), cells were stained according to the kit’s introductions and the percentage of dead cells/total cells was calculated by high-content analysis (PerkinElmer, USA). Briefly, cell images were captured by an Operetta CLS (PerkinElmer, USA). In each well, 9 image fields were captured at the same exposure time, and at least 200 cells per field were calculated for effective data output. The calculations were performed automatically by the software Harmony 4.1 (PerkinElmer, USA) with the recommended conditions of the live/dead assay in this system. Live/dead assays were performed to distinguish between live and dead cells. This kit (Solarbio, China) provides a two-colour fluorescence cell viability reagent including Calcein-AM and propidium iodide dye to determine the intracellular esterase activity and plasma membrane integrity. The detailed method has been described previously.19

2.9 TEM analysis
The cells were incubated with 100 μg mL−1 PS M/NPLs for 24 h and harvested by centrifugation at 200 g for 5 min. Then, the treated samples were centrifuged repeatedly and washed thrice with PBS. Meanwhile, the supernatant was removed. The harvested cell pellets were fixed overnight with 2.5% glutaraldehyde. The cells then were washed three times with PBS (pH 7.0) containing 250 mM sucrose for 10 min/time and post-fixed in 1% osmium tetroxide in 0.1 M PBS (pH 7.0) for 2 h. The specimens were then dehydrated in a series of graded alcohol solutions (50%/70%/80%/90%/100%) and embedded in araldite at different temperatures (37 °C/45 °C/70 °C) for 24 h. Ultra-thin sections of approximately 100 nm were cut with an ultramicrotome, picked up on copper grids and observed with a JEOL CX100 TEM at 80 kV.

2.10 Flow cytometry analysis
Flow cytometry was used to detect the cellular internalization of PS M/NPLs. After incubation with a 100 μg mL−1 concentration of PS M/NPLs for 24 h, the cells were digested with 0.25% (w/v) trypsin-EDTA solution (Wistet, China). To completely remove the trypsin-EDTA and PS M/NPLs, the treated samples were centrifuged repeatedly and washed thrice with PBS and then measured using a flow cytometer (BD, USA) equipped with forward scatter and side scatter. The intensity of the forward-angled scattered light was related to the size of the cell. For the same spherical cells, the intensity increased with the cross-sectional area of cells. The intensity of the side-scattered light was related to the intracellular density, which is mainly used to obtain relevant information about the subtle structure inside the cell. A quantitative analysis of the intensity of the side-scattered light by side scatter was performed to determine the internalization levels of the PS M/NPLs.40

2.11 Trans endothelial electrical resistance (TEER) measurements
Caco-2 cells cultured for about 3 weeks in 12-well Transwell filters (polyester, 0.4 μm pores, Costar) could be used as the cell model of the intestinal epithelial TJ barriers, as described previously.41,42 Caco-2 cells were plated at 2 × 105 cells per well in the apical chamber; meanwhile, 0.5 mL and 1.5 mL of the medium was added to the apical and basal chamber, respectively. The medium was changed, and the TEER was measured with a Millicell-ERS (Millipore, USA) every other day. About 24 days after confluence, when the Caco-2 monolayers reached the epithelial maximum resistance of 550–600 Ω cm−1, the cells were incubated at a concentration of 200 μg mL−1 with PS
M/NPLs for 24 h. TEER was calculated as follows: TEER = \( \frac{R_{\text{cell}} - R_{\text{blank}}}{A} \times A \), where TEER is expressed in Ω cm².

2.12 Paracellular permeability test
Caco-2 cells were cultured on a transwell membrane by forming closely contacted monolayers. The monolayers were then incubated with 200 μg mL⁻¹ various PS M/NPLs for 24 h. After treatment, cells were rinsed twice with PBS and incubated in PBS containing 0.5 mg mL⁻¹ fluorescein sodium in the apical compartment of the transwells. Fluorescein sodium was assessed by taking 100 μL from the basolateral compartment. At the same time, the same volume of PBS was added after 30, 60, 90 and 120 min. The fluorescence signal was monitored at a wavelength of 505 nm by a microplate spectrophotometer (Tecan, Durham, USA). The concentrations of fluorescein sodium were determined using a standard curve generated by the intensities of different concentrations of fluorescein sodium. The permeability coefficient Capp value was calculated as \( \text{Capp} = \frac{\text{dQ/dt}}{(A \times C_{0})} \), where \( \text{dQ/dt} \) is the amount of fluorescein sodium translocation per unit of time, \( A \) is the membrane surface area and \( C_{0} \) is the initial apical concentration. 1% Triton X-100 was used as the positive control.

2.13 Real-time PCR, Elisa, Western blotting and immunofluorescence analysis
The expression levels of cellular TJ proteins were quantified by previously described methods. Briefly, the samples were lysed in RIPA buffer with protease inhibitors (Beyotime, China) on ice for 20 min. Then, the protein concentrations were determined using the BCA method (Thermo Scientific, USA) before detection. In the immunofluorescence analysis, the monolayer of cells on the transwell membrane was rinsed twice in PBS, fixed with 4% paraformaldehyde for 1 h, permeabilized with 0.1% Triton in PBS for 20 min, blocked with 10% goat serum in PBS for 1 h followed by incubation with the primary antibody for at least 2 h, washed three times with PBS and incubated with a fluorescence-labelled secondary antibody (1:500 dilution for 1 h). The coverslips were mounted after washing three times with PBS. Images for the morphological analyses were acquired with confocal microscopy (UltraView VoX, PerkinElmer, USA) with an oil immersion objective.

2.14 Statistical analysis
The results are expressed as the mean ± standard deviation (SD) of at least three independent experiments. The statistical analyses were performed by unpaired Student's t-tests with SPSS 19.0 software, and a P value of <0.05 was considered to indicate a significant difference.

3. Results

3.1 Characterization of PS micro- and nanoparticles
PS M/NPLs were purchased from the Tianjin BaseLine Chromtech Research Center (Tianjin, China) and stocked in aqueous solution. For simplicity, the 70 nm NPLs of pristine and different functionalized particles are referred to as nano PS, nano PS-COOH and nano PS-NH₂, and the 5-μm MPLs are referred to as micro PS, micro PS-COOH and micro PS-NH₂. The size distributions, polydispersity indexes (PDI), zeta-potentials and morphological images of the PS M/NPLs are summarized in Table S1† and Fig. 1. The characterization results revealed that the size distribution and surface charges measured by dynamic light scattering (DLS) matched the claimed sizes of the supplier. Generally, the PS M/NPLs exhibited uniform spherical morphologies and optimal dispersion in deionized water. The PDI of micro PS-NH₂ was higher than 0.3, indicating that the micro PS-NH₂ are prone to be in the agglomerated state in solutions. The high absolute zeta-potentials of PS-COOH and PS-NH₂ indicate that these particles are more stable in aqueous solution. In the presence of DMEM, gastric simulated fluid (GSF) and intestinal simulated fluid (ISF), the PS M/NPLs tended to aggregate differently. The DLS data showed that the positively charged micro- and nanoparticles (PS-NH₂) aggregated much more than the pristine and negatively charged micro- and nanoparticles (PS-COOH) in DMEM, gastric simulated fluid and intestinal simulated fluid (Table S1†). The Fourier transform infrared (FTIR) spectra in the range of 400–4000 cm⁻¹ were used to identify the possible variation in the functional groups (Fig. S1†). The PS-COOH samples exhibited characteristic peaks at 1601.2 cm⁻¹, 1452.3 cm⁻¹ and 698.4 cm⁻¹, which are caused by the rigid vibration of the monosubstituted benzene constituent of the polymer. The peaks at 3025.8 cm⁻¹ and 3082.3 cm⁻¹ correspond to the stretching vibration of the C-H bond of the benzene ring itself. The samples also have a characteristic peak at 2922.1 cm⁻¹, which is caused by the stretching vibration of the C-H bond of –CH₂–. The peaks at 1452.3 cm⁻¹ and 1493.1 cm⁻¹ correspond to the bending vibration of –CH₂–. In addition, the PS-NH₂ samples have a characteristic peak at 3405.9 cm⁻¹, which is due to the vibration of the aromatic amine.

3.2 Toxicity of the PS micro- and nanoparticles on mice after gavage exposure
PS M/NPLs were administrated to C57/B6 mice by gavage at 0.2 mg per kg per day (low dosage) and 2 mg per kg per day (high dosage) for 28 days. These animal doses are about 0.0184 mg per kg per day (low dosage) and 0.184 mg per kg...
per day (high dosage) for the human equivalent doses after calculation using a well established method. By comparison, the exposure dose of an adult (70 kg) will reach about 2 mg per kg per day when they live in a high microplastic pollution area, i.e., 30–50 mg L$^{-1}$ in Taihu Lake. The oral administration of nano PS led to a significant decrease in body weight compared with the control and micro PS groups (Table S2†). The body weight data illustrated that the surface amination modification has high toxicity to mice in both the low- and high-dosage exposure groups. The levels of serum alkaline phosphatase (ALP), aspartate aminotransferase (AST), total bilirubin (T-Bil), creatine kinase (CK), r-glutamine transferase (r-GT) and serum creatinine (SCr) increased significantly after exposure to 2 mg kg$^{-1}$ PS-NH$_2$ relative to the control group, indicating the systemic functional damage to the liver, heart and renal system. r-GT is related to the metabolism of the organism, and its significant changes were mostly found in the high-dosage-exposure groups. Furthermore, a significant increase in CK was also found in the low-dosage-exposure groups, which means that long-term exposure to M/NPLs is a potential hazardous factor for myocardial injury (Table S3†).

The histopathologies of the heart, liver, spleen, lung, kidney, stomach, small intestine (duodenum, jejenum) and large intestine (colon) were analyzed after haematoxylin-eosin staining (Fig. 2A and Fig. S2†). After correlation with the blood biochemical analysis results, the histopathological images indicated that obvious injuries occurred in the livers, kidneys and spleens of the mice in the high-dosage-exposure groups (Fig. S2†). These injuries may be partly related to the high immunoreactivity which was demonstrated by the accumulation of cytotoxic T lymphocytes in the spleen (Fig. S3†). Further tests indicated that all of the observed systemic organ toxicities may result from the injuries to the GI tract in the groups with high-dosage exposure to PS M/NPLs. The morphologies of the stomachs, duodena, jejuna and colons were significantly injured after exposure to PS-NH$_2$. Specifically, the crypts were damaged, the wrinkles and villi of the intestine disappeared, and the intestinal, stomach, and mucosa walls became thin and had obvious inflammatory exudation layers after exposure to PS-NH$_2$ (Fig. 2A). Simultaneously, the mRNA and protein levels of TJ proteins, ZO-1, Claudin-1 and Claudin-3 were differentially modulated by the various PS M/NPLs in the intestine. Micro/nano PS and micro PS-COOH showed less impact on the expressions of TJ proteins, while nano PS-COOH moderately decreased and micro/nano PS-NH$_2$ dramatically decreased their expression levels (Fig. 2B and C). These results

Fig. 2 Toxic effects on the digestive systems in mice after gavage exposure to PS M/NPLs for 28 days at a dosage of 2 mg per kg per day. (A) Morphological changes in the tissues of the digestive system in mice. The black arrows show the damaged tissues with the wall of intestines and stomachs becoming thin, the intestinal follicles of the duodenum atrophying, and the structure disintegrating. (B) mRNA expression levels. (C) Protein expression levels determined by ELISA. Data are expressed as mean ± SEM. $n = 6$ for mRNA levels and $n = 4$ for protein quantification. *$P < 0.05$, **$P < 0.01$. 

Published on 07 April 2021. Downloaded on 6/21/2021 2:55:07 PM.
indicate that the oral administration of PS M/NPLs, especially micro/nano PS-NH₂, has obvious toxic effects on the GI tract, that is, causing prominent injuries to the epithelial systems of the stomach and intestine.

3.3 PS M/NPLs moderately influence the integrity and function of the gut epithelial barrier in cell model

To explore the potential mechanisms of the PS M/NPLs disrupting the gut tract, we first investigated whether the PS M/NPLs can be found in the Caco-2 intestinal epithelial cells. The intracellular location and trafficking of the PS M/NPLs were monitored by TEM and flow cytometry (Fig. 3). All of the pristine and surface-modified NPLs could be detected in the cytoplasm and were distributed in the cellular lysosome, whereas only the PS-COOH MPLs were found in the cellular cytoplasm. These results suggested that the PS M/NPLs can be endocytosed in intestinal epithelial cells, implying that the PS M/NPLs may deteriorate gut epithelial function directly.

We assessed the cytotoxicity of the PS M/NPLs in Caco-2 intestinal cells. The CCK-8 and the live/dead cytotoxicity assay revealed that all of the PS M/NPLs had no evident dose-response toxic effect on the Caco-2 cells up to 200 μg mL⁻¹ (Fig. S4 and S5†). We then evaluated the influence of the PS M/NPLs on the physiological functions of the gut epithelia cells in vitro in the monolayer cell cultures. The TEER test shows that the Caco-2 monolayers can have TEER values higher than the epithelial resistance of 400 to 550 Ω cm⁻² from 3 weeks of consecutive culture (Fig. S6†). Then, the disruption ability to the epithelial TJ proteins was tested using this cell model after incubation with 200 μg mL⁻¹ PS M/NPLs for 24 hours. It was demonstrated that exposure to nano PS-NH₂ induced a significant decrease in the TEER values while its effect was quite weak when compared to 1% Triton X-100 (Fig. 4A). Similarly, permeability tests showed that exposure to micro/nano PS and micro/nano PS-COOH did not increase the paracellular permeability (Fig. 4B). Western blot analysis of the TJ proteins (occludin, claudin-1 and ZO-1) and immunofluorescence of occludin and ZO-1 also reveal that micro/nano PS and micro/nano PS-COOH had little influence on gut TJ protein levels (Fig. 4C and D). Exposure to aminated PS, especially nano PS-NH₂, led to significant alterations in the paracellular permeability and TJ protein levels (Fig. 4B–D). Collectively, these results indicated that exposure to a high concentration of PS M/NPLs (200 μg mL⁻¹) had little (micro/nano PS and micro/nano PS-COOH) or moderate (nano PS-NH₂) impact on the integrity and function of the gut epithelial barrier, which is in conflict with their obvious in vivo damage to the gut tract, especially for nano PS-NH₂. Therefore, other factors may participate in the toxicity of the PS M/NPLs.

3.4 Oral administration of PS M/NPLs shifts gut microbiota in mice

The important roles of gut microbes in the maintenance of host health have attracted much attention in the past decade and several investigations have reported the modulating effect of microplastics on the structure of gut microbiota. However, there is little evidence linking the changes in gut microbial communities and the toxicity of microplastics to the GI tract. We first checked the influence of PS particles on the whole structure of the gut microbiota. Twenty eight day exposure to various PS M/NPLs led to a moderate decline in the diversity of the gut microbiota, as indicated by the Shannon and Simpson indexes (Fig. 5A and B). Exposure to PS M/NPLs that are modified with either carboxyl or amino groups resulted in a more decreased diversity, suggesting that chemical modification may aggravate the impact of PS M/NPLs on gut microbial diversity. The unmodified PS MPLs and amino-modified PS MPLs resulted in a greater decrease in microbial diversity than the nano-sized ones, while the carboxyl-modified PS M/NPLs resulted in minimal difference. Principal coordinates analysis (PCoA) and hierarchical clustering analysis showed that while the unmodified nano PS had little influence on the gut microbiota, the unmodified MPLs and M/NPLs modified by carboxyl or amino groups largely shifted the overall structure of the gut microbial community (Fig. 5C and D). Generally, the impact of the various types of M/NPLs on gut bacteria was as follows: amino-M/NPLs > carboxyl-M/NPLs > unmodified M/NPLs. In terms of the particle size, the micro-sized M/NPLs more significantly shifted the overall structure of the gut microbial community compared to the nano-sized M/NPLs (Fig. 5C and D).

We further checked the impact of the PS M/NPLs on the abundance of individual taxa to identify the main bacteria that...
respond to PS M/NPLs exposure. The taxon-based analysis also revealed that PS M/NPLs markedly modulated the gut bacteria. We compared the gut microbial composition among different groups at the phylum and genus levels. As shown in Fig. S7A† treatment with various PS M/NPLs had little influence on the relative abundances of Firmucutes and Bacteroidetes, two main phyla in the gut microbiota that account for more than 95% of the total gut bacteria. However, exposure to amino-M/NPLs in both micro- and nano-sizes resulted in a marked increase in Proteobacteria, a known phylum containing multiple opportunistic pathogens (Fig. S7B†). In addition, amino-MPLs also led to a significant increase of Verrucomicrobia at a high concentration (Fig. S7B†). Fig. S8† displays the abundance of changes to the top 21 genera among the different groups. At the genus level, various PS M/NPLs decreased the abundance of most main genera but generally promoted the growth of Lactobacillus. Two genera, the [Eubacterium]_xylanophilum_group and the Lachnospiraceae_NK4A136_group, were significantly decreased by most types of PS M/NPLs, and four known short-chain fatty acid (SCFA)-producing genera, i.e., Ruminiclostridium_9, Lachnoclostridium, Anaerotruncus and Ruminiclostridium, were significantly decreased by the aminomodified M/NPLs in both micro- and nano-sizes. In general, the chemically-modified PS M/NPLs had a more potent impact on gut bacteria than the unmodified M/NPLs, and the amino-modified PS M/NPLs displayed the most pronounced influence. As for the particle size, the micro-sized PS generally exhibited a more significant impact on the various genera than the nano-sized counterparts.

3.5 The M/NPLs-modulated gut bacteria were closely related with gut tract tight junctions in mice

To find the key phenotypes that were correlated with the toxicity of the PS M/NPLs, we generated microbial co-occurrence networks using the SparCC method. \(^5\) A total of 71 genera with \(R\) values of >0.5 and \(P\) values of <0.01 were displayed in the co-occurrence network (Fig. 6A). They can be obviously clustered into three groups based on their correlation and abundance distribution among the different treatments. Thirty-one genera including Anaerotruncus, Ruminiclostridium_5, Roseburia, Ruminiclostridium, Butyricicoccus, etc. were decreased by the PS M/NPLs, especially the amino-PS M/NPLs (Fig. 6B). All of these PS M/NPLs-decreased genera are known SCFA producers, and many of them such as Roseburia, \(^5\) Butyricicoccus and Anaerotruncus have been proven to be beneficial for the TJs of the gut epithelium. It is well documented that gut microbiota-derived SCFAs are effective in maintaining intestinal barrier function. \(^5\) On the other hand, five genera, Parasutterella, Romboutsia, Serratia, Achromobacter and Turicibacter were enhanced by the PS-NH\(_2\) M/NPLs (Fig. 6B). These genera belong to the Gram negative bacteria and contain multiple opportunistic pathogens and their overgrowth often accompa-
the barrier function of the gut tract. Inflammation by the modulation of gut microbes, thus destroying the barrier function of the gut tract. These results provide a valuable hint that the long-term exposure to PS M/NPLs could lead to epithelial injury in the digestive system. It was demonstrated that PS M/NPLs can lead to the dysbiosis of the intestinal flora, with a significant decrease in the content of beneficial bacteria but a high increase in harmful bacteria which has been proven to be harmful to the intestinal TJs. Thus, exposure of PS M/NPLs to the digestive system plays an important role in the intestinal epithelial barrier. Furthermore, the internal injuries caused by the toxicity of the PS M/NPLs will lead to systemic toxicity at the whole organism level, such as loss of body weight, influence on serum biochemical markers and organ toxicity.

In order to analyze the impact of PS M/NPLs on human health, we conducted in vitro testing using Caco-2 cells by exposing them to different surface-modified PS M/NPLs. We found that the toxicity of non-modified PS M/NPLs to the human body is relatively weak. Comparatively, the in vivo toxicity of carboxyl modified MNPs is moderate, while the toxic damage of amino modified PS M/NPLs to animals is quite strong. It could be concluded that amino-modified PS M/NPLs are more harmful to the human body and should be paid more attention.

Growing evidence has shown that environmental factors may affect host health by regulating intestinal flora. Within this research, the most common observation was that the MPLs induced dysbiosis of the gut microbiota, leading to microbial communities which were distinct from the controls without exposure treatments. But these studies did not correlate the changes in gut flora with the adverse effects of the MPLs from the point of the view of a basic mechanism. In our study, we analyzed the co-occurrence network of the intestinal flora for the first time, and investigated the adverse effect of PS M/NPLs in regulating intestinal flora as a whole. The bacteria that the PS M/NPLs reduced were mostly short-chain fatty acid-producing bacteria, many of which have been shown to help improve intestinal TJs. The bacteria that are upregulated by PS M/NPLs are almost always pathogenic bacteria and have destructive effects on intestinal epithelial function. It has been well documented that gut microbe-derived metabolites play important roles in the maintenance (SCFAs) or destruction (LPS) of the gut barrier function. Thus, our analysis provides a clear regulatory network of intestinal flora and correlates changes in the intestinal flora with the disruption of the intestinal barrier function by PS M/NPLs. Although further investigations are needed get more solid evidence, these results have provided an important hint that intestinal flora imbalance is at least partially involved in the adverse effects of exposure to PS M/NPLs on the host organism.

The exposure dosages in this study are based on data on MPLs in high-pollution areas. Thus, the high dosage of 2 mg per kg per day currently used is probably a high estimation for most people who live in environments with a normal level of M/NPLs pollution. Our research found that one-tenth of the high dosage is enough to have a significant toxic influence on mice. For humans, no one can escape the expectation of the lifelong accumulation of M/NPLs based on the current situation of MPLs pollution. The in vitro testing results from human Caco-2 cells show that PS M/NPLs could directly influ-

4 Discussion

In expectation of the high accumulation of plastic debris in the environment, M/NPLs are sure to cause great harm to ecosystems and humans. Several studies have shown that MPLs contribute to injuries to intestinal tissues and the disturbance of intestinal flora. However, whether there is an intrinsic connection between the observed adverse outcomes is unknown. In this study, we tested the in vivo toxicity of PS M/NPLs in mice, focusing on the injuries to the intestinal tissues and the influences on the intestinal flora. Our results show that long-term exposure to PS M/NPLs could lead to epithelial injury in the digestive system.
ence human intestinal epithelial cells with two possible adverse outcomes: particle uptake in the cytoplasm and/or a ruined intestinal barrier. In this research, only carboxyl-modified MPs were observed by TEM. This may be due to the reason that large-size PS MPLs may have a mass effect on organelles in the cytoplasm, which causes obvious physical damage to cells which have endocytosed them.\textsuperscript{64,65} Furthermore, surface charges play a predominant role in the cell death induced by the interaction of the MPLs with cells with respect to the size and shape of the particles.\textsuperscript{43,66,67}

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**Fig. 6** SparCC correlation networks among genera (A) and the impact of various MNPs on the abundance of typical genera (B). Nodes correspond to genera, and connecting edges indicate correlations between them. Only nodes with negative or positive correlations, with values of less than $-0.50$ (blue) or larger than $0.50$ (red), are represented. The area of each node indicates the accumulated abundance of the genus in all groups, and the proportions of each group are displayed in different colours.
means that M/NPLs that have different properties may undergo different internalized pathways which lead to different toxic impacts on the organism. It is clear that even short-term exposure to amino-modified PS particles, such as the 24 h used in this research, could lead to the collapse of the intestinal epithelial TJs of the Caco-2 cells. Thus, it is reasonable to propose that the accumulated injuries to the digestive tract after chronic exposure would lead to systemic toxicity in the organism.

5 Conclusions

In this study, our results have demonstrated that long-term exposure to PS M/NPLs, especially amino-modified PS M/NPLs, causes systemic damage to the body as a result of the impaired intestinal epithelial barrier function. The intestinal flora imbalance caused by the PS M/NPLs plays an important role in the impaired intestinal barrier.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The authors acknowledge the financial support received from the National Natural Science Foundation of China (21777036, 81973217, 81673663) and the Ministry of Science and Technology of China (2016YFA0201600).

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