Chemosphere 244 (2020) 125492

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

# Polyethylene microplastics affect the distribution of gut microbiota and inflammation development in mice

Boqing Li [a, 1], Yunfei Ding [a, 1], Xue Cheng [a, 1], Dandan Sheng [b], Zheng Xu [a], Qianyu Rong [a], Yulong Wu [a], Huilin Zhao [a], Xiaofei Ji [a], Ying Zhang [a, *]

[a] School of Basic Medical Sciences, Binzhou Medical University, Yantai, 264003, China
[b] Yantai City Hospital for Infectious Diseases, No.62, Huanshan Road, Yantai, 264003, China

## HIGHLIGHTS

- Polyethylene microplastics affected the composition and diversity of gut microbiota.
- Polyethylene microplastics increased the secretion of IL-1α in serum.
- Polyethylene microplastics decreased the Th17 and Treg cells among $CD4^+$ cells.
- High-concentration polyethylene microplastics induced small intestinal inflammation.

## ARTICLE INFO

*Article history:*
Received 25 August 2019
Received in revised form
28 October 2019
Accepted 26 November 2019
Available online 27 November 2019

Handling editors: Tamara S. Galloway

*Keywords:*
Microplastics
Polyethylene
Gut microbiota
Inflammation response
Mice

## ABSTRACT

Environmental pollution caused by plastics has become a public health problem. However, the effect of microplastics on gut microbiota, inflammation development and their underlying mechanisms are not well characterized. In the present study, we assessed the effect of exposure to different amounts of polyethylene microplastics (6, 60, and 600 μg/day for 5 consecutive weeks) in a C57BL/6 mice model. Treatment with a high concentration of microplastics increased the numbers of gut microbial species, bacterial abundance, and flora diversity. Feeding groups showed a significant increase in *Staphylococcus* abundance alongside a significant decrease in *Parabacteroides* abundance, as compared to the blank (untreated) group. In addition, serum levels of interleukin-1α in all feeding groups were significantly greater than that in the blank group. Of note, treatment with microplastics decreased the percentage of Th17 and Treg cells among $CD4^+$ cells, while no significant difference was observed between the blank and treatment groups with respect to the Th17/Treg cell ratio. The intestine (colon and duodenum) of mice fed high-concentration microplastics showed obvious inflammation and higher TLR4, AP-1, and IRF5 expression. Thus, polyethylene microplastics can induce intestinal dysbacteriosis and inflammation, which provides a theoretical basis for the prevention and treatment of microplastics-related diseases.



## 1. Introduction

Microplastics pollution has become one of the four major global environmental problems in parallel with global climate change, ozone depletion, and ocean acidification (Galloway and Lewis, 2016). Microplastics refer to plastic debris, particles or thin films with a diameter less than 5 mm, which can be divided into primary and secondary microplastics (Thompson et al., 2004). Primary microplastics refer to the industrial products of plastic particles discharged into the ocean through rivers and sewage treatment plants, and secondary microplastics are small fragments gradually formed by large plastic waste fragments in the environment. The annual output of plastic waste discharged to the ocean may reach from 4.8 to 12.7 million tons (Jambeck et al., 2015), and this plastic waste will continuously break into microplastics. Due to their small size, microplastics can easily be swallowed by organisms and accumulated *in vivo*, which is of biological concern (Teuten et al., 2009). Microplastics can enter the human body in many ways, affecting human health. Firstly, they can be transmitted through the food chain (web) and eventually enter the human body due to the

* Corresponding author. School of Basic Medical Sciences, Binzhou Medical University, 346# Guanhai Road, Yantai, 264003, China.
*E-mail address:* zhangying99g99@163.com (Y. Zhang).
[1] These authors contributed equally to the paper.

https://doi.org/10.1016/j.chemosphere.2019.125492
0045-6535/

accumulation of microplastics in organisms (Setälä et al., 2014). Secondly, eating sea salt also increases the risk of human exposure to microplastics (Yang et al., 2015). Moreover, the source of drinking water on which human beings depend has also been affected by microplastics (Kosuth et al., 2018). Although microplastics pollution has attracted the attention of the international community, most of the studies have focused on the migration and distribution of microplastics in the environment, and their effects on marine organisms. There is a lack of research on the health risks of human intake of microplastics.

Microbes have a close relationship with human beings; diversity of the gut microbiome is crucial for human health (Costello et al., 2009). Imbalance of the microbial community has been demonstrated in patients with inflammatory bowel diseases (Frank et al., 2007). Previous studies have explored the significant effects of polystyrene (PS) on the composition and structure of intestinal microflora in mice (Lu et al., 2018; Jin et al., 2019). Different compositions of the intestinal tract microbiome may have different effects on immune function. Specific bacteria have the ability to induce the differentiation of Treg cells and modulate the inflammatory process by their effects on pro-inflammatory or anti-inflammatory cytokines, such as interleukin (IL)-8 and IL-10 (Kamada et al., 2013; Underwood, 2014). Gut microbes such as Firmicutes (Obrenovich et al., 2017) and *Bifidobacterium infantis* (El Aidy et al., 2012) have been shown to promote the induction of Treg cells and secretion of the anti-inflammatory cytokine, IL-10. The tight control of the ratio of Treg/Th17 cells by gut microbiota helps maintain the normal immune response. Intestinal flora can also activate and regulate signal transduction pathways related to intestinal mucosal immune function, such as the TLR pathway, which constitute an intestinal defense mechanism to jointly resist the invasion of foreign substances (Kamada et al., 2013).

The effects of microplastics pollution on human health are not well characterized. Therefore, it is important to explore the effects of microplastics on intestinal microflora composition and inflammation development in mice as mammalian models. Microplastics particles mainly include polyethylene (PE), polypropylene, polystyrene and polyester. In China, PE microplastics are the main type of microplastics, regardless of whether the microplastics are in the marine environment, freshwater environment, or soil (Zhao et al., 2018; Wu et al., 2018; Luo et al., 2018). We hypothesized that PE microplastics exposure will change the numbers of gut microbial species, bacterial abundance, and flora diversity, which affect the secretion of cytokines and the percentage of Th17 and Treg cells among $CD4^+$ cells. Specifically, we predicted that alteration of gut microbiota would induce intestinal inflammation through activation of TLR4 signaling.

## 2. Materials and methods

### 2.1. Feed preparation

Mouse feeds were purchased from the Beijing keao Xieli Feed Company (Beijing, China). Male C57BL/6 mice were purchased from the Jinan Pengyue Laboratory Animal Breeding Company (license key: SCXK (Lu) 20140007) (Jinan, China). Clear PE microplastics (cat. no. CPMS-0.96) (diameter: 10–150 μm) were purchased from the Cospheric Company. Animal use was approved by the animal ethics committee. Feed formula was prepared as follows: 0.02, 0.2, or 2 g microplastics were dissolved in 10 kg of basal feed to obtain a concentration of 2, 20, or 200 μg $g^{-1}$ microplastics, respectively.

Eighty 5-week-old SPF grade C57BL/6 male mice were divided into four groups (n = 20 for each group). The blank group was fed a basal feed, while the other three groups were fed special feeds containing 2, 20, and 200 μg $g^{-1}$ microplastics, respectively, for 5 consecutive weeks. Feed consumption per mouse was calculated as 3 g per day. Therefore, the amount of PE microplastics consumed by each mouse every day was 6, 60, or 600 μg, respectively.

### 2.2. Microbial DNA extraction and structural analysis

Genomic DNA was extracted from the fecal samples of mice in all groups by CTAB method. The diversity of microorganisms was identified by V4 region primers 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′) (Liao et al., 2018). A DNA library was constructed using the ThermoFisher kit and DNA samples were sequenced using Life-Ion S5 (Thermofisher Company).

Sequence reads processing was performed using QIIME (version 1.9.1) and included additional quality trimming and demultiplexing. Sequences were assigned to the same operational taxonomic units (OTUs) if they had greater than or equal to 97% similarity. The Venn diagram based on OTUs, alpha diversity (Chao1 and Shannon indices), beta diversity using weighted UniFrac, and top 12 genera were calculated by QIIME (version 1.9.1) and displayed using R software (version 2.15.3).

### 2.3. Examination of cytokines in mouse serum

Mouse serum levels of cytokines (IL-1α, G-CSF, IL-2, IL-5, IL-6, IL-9, IP-10, and RANTES) were determined using the mouse cytokine/chemokine magnetic bead panel 96-well plate assay (Millipore Corp, Billerica, MA, USA), according to the manufacturer's recommendations. Measurements were performed with the Luminex Xmap MAGPIX system (Luminex Corp, Austin, TX, USA) (Schirmer et al., 2016). Absorbance values were detected using a microplate reader.

### 2.4. Determination of mouse Th17/Treg phenotype

Intracellular cytokine staining of splenocytes was performed as described elsewhere (Cui et al., 2018). Mouse Th17/Treg Phenotyping Kit (560767, BD Pharmingen) was used for this experiment. APC anti-mouse CD4 and FITC anti-mouse IL-17A were used for Th17 cell staining, whereas APC anti-mouse CD4, PE anti-mouse CD25, and Alexa Fluor 488 anti-mouse Foxp3 were used for Treg staining. Signals were detected by flow cytometry (FACSCalibur). Data analyses were performed using Cell Quest-Pro software.

### 2.5. Assessment of intestinal inflammation in mice

The colon and duodenum tissues of mice were removed and fixed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E) to evaluate the presence of inflammatory cells, which were scored histologically (from 0 to 3). According to the occurrence of intestinal epithelial inflammation, scores were as follows: 0: normal intestinal epithelial cells; 1: mild, villous core separation, and no other abnormalities; 2: moderate, villous core separation, submucosal edema, and epithelial exfoliation; and 3: severe, denudation of epithelium with loss of villi, full-thickness necrosis, or perforation (Nadler et al., 2000).

### 2.6. Immunofluorescence staining

The colon and duodenum tissues of mice were frozen and sliced. The slides were blocked with 10% goat serum for 1 h, and incubated with the following primary antibodies: mouse anti-TLR4 (Abcam, Cambridge, UK), rabbit anti-AP-1 (Proteintech, Rosemont, IL, USA),

and mouse anti-IRF5 (Abnova, USA), diluted with phosphate-buffered saline containing 0.1% Triton X-100 at 4 °C overnight. Subsequently, the slides were incubated with goat anti-mouse or goat anti-rabbit Dylight488-conjugated secondary antibody (Abbkine, USA) and DAPI to stain cell nuclei. Sections were evaluated using laser scanning confocal microscopy (Zeiss MIC-SYSTEM). The average fluorescence intensity measuring the fractions of TLR-1, AP-1, and IRF-5 was determined with randomly selected images ($n \geq 30$) using the confocal microscopy software ZEN 2.5 lite.

### 2.7. Data analysis

The alpha and beta diversity indices among groups were assessed using the Wilcoxon rank-sum test using the agricolae package in the R software (version 2.15.3) (Brannock and Halanych, 2015). Analysis of similarities (ANOSIM) was performed using the anosim function in the R vegan package. Between-group differences in top 12 abundant genera were assessed using the MetaStat method in R software. The mouse cytokines and Th17/Treg phenotype were compared with ANOVA analysis using the R package (Ellis et al., 2017). The results were expressed as mean ± standard deviation. *P*-values < 0.05 were considered statistically significant.

## 3. Results

### 3.1. Effects of PE microplastics exposure on gut microbial community distribution

The common and unique OTUs of different samples (groups) were summarized according to the result of OTU clustering analysis (Fig. S1). The number of OTUs in the blank (c), 6 μg (t06), 60 μg (t60), and 600 μg (t600) groups were 1457, 1406, 1456, and 1996, respectively. The common OTU number of these groups was 1286. The 600 μg group had the most specific OTU number; i.e., 491; this suggested that treatment with a high concentration of microplastics resulted in a significant increase in the number of species of intestinal microbes (maximum number of unique microbial species).

The changes in alpha diversity of the intestinal microbiome of mice after feeding with different concentrations of microplastics were analyzed using Chao1 and Shannon indices. Chao1 indices for the c, t06, t60, and t600 groups were 905.85 ± 54.92, 884.47 ± 61.45, 916.16 ± 62.46, and 1049.40 ± 160.64, respectively (Fig. 1A). Wilcoxon test revealed a significant difference between the t600 group and the other groups with respect to Chao1 index ($P < 0.01$), while no significant difference was observed between the other three groups. These results indicated a significant increase in bacterial abundance in mice fed a high concentration of microplastics.

The Shannon indices for the c, t06, t60, and t600 groups were 6.87 ± 0.23, 6.88 ± 0.37, 6.91 ± 0.40, and 7.06 ± 0.37, respectively (Fig. 1B). Wilcoxon test revealed a significant difference between the t600 and c groups in this respect ($P < 0.05$); however, no significant difference was observed among the t600 group and the other two groups. The results showed that the flora diversity increased significantly in mice fed a high concentration of microplastics.

The beta diversity index is presented using UniFrac (Fig. 1C). The beta diversity index of the c group was significantly different from those of the t06 and t600 groups ($P < 0.01$). The difference in the beta diversity index between the t06 and t60 groups was also statistically significant ($P < 0.01$). The beta diversity index of the t60 group was significantly different from that of the t600 and c groups ($P < 0.05$). However, no significant difference was observed between the t06 and t600 groups in this respect ($P = 0.36$). These results showed an increase in intestinal microbial diversity in mice fed with PE microplastics.

To display the proportion of different species at the phylum level, the column diagram was generated based on the relative abundance of species (Fig. S2). The results showed that most microorganisms in the fecal samples of mice belonged to nine phyla (Bacteroidetes, Firmicutes, Proteobacteria, Melainabacteria, Actinobacteria, Deferribacteres, Tenericutes, Verrucomicrobia, and Chloroflexi), and other microorganisms belonged to unidentified bacteria and others. Bacteroidetes and Firmicutes were the two largest categories in the fecal samples of mice. The percentage of Bacteroidetes and Firmicutes in the c group was significantly higher ($P < 0.05$) and lower ($P < 0.01$) than those of the t60 and t600 groups. There were lower percentages of Melainabacteria ($P < 0.05$) in the c group compared with the t06, t60, and t600 groups. The differences of Firmicutes and Deferribacteres between the t06 and t60 groups were also statistically significant ($P < 0.05$). The percentage of Actinobacteria in the t06 group was significantly different from that of the t600 group ($P < 0.05$). There was a significant difference in Deferribacteres between the t60 and t600 groups ($P < 0.05$).

The boxplot was drawn according to the distribution of species with different abundance at the genera level. The top 12 abundant genera are summarized in Fig. 2. The results showed an obvious

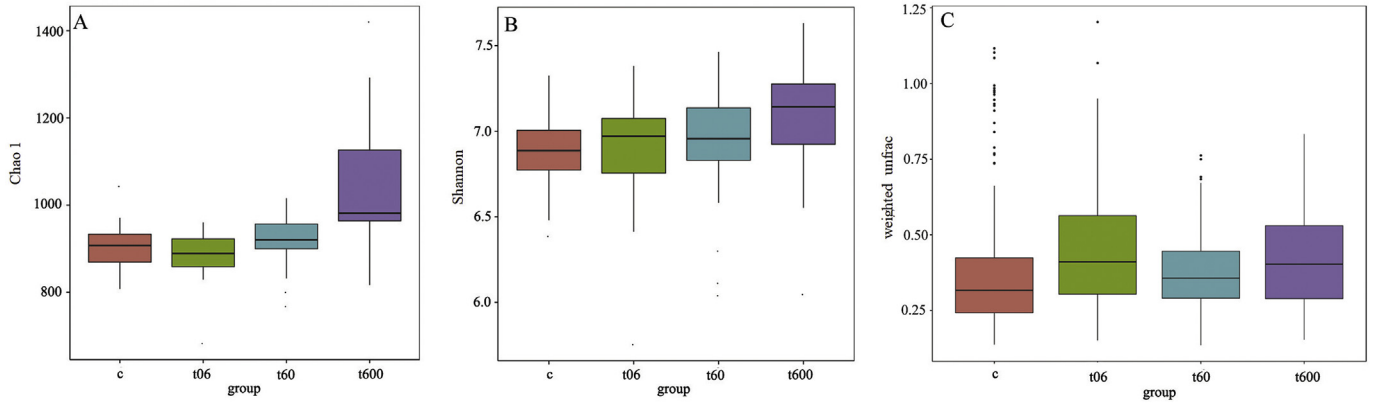


**Fig. 1.** Chao1 diversity index (A), Shannon index (B), and UniFrac beta diversity metrics (C) in the c, t06, t60, and t600 groups. The boxplot depicts the interquartile range and the line inside the box denotes the median. c, blank group; t06, 6 μg microplastics treatment group; t60, 60 μg microplastics treatment group; and t600, 600 μg microplastics treatment group.

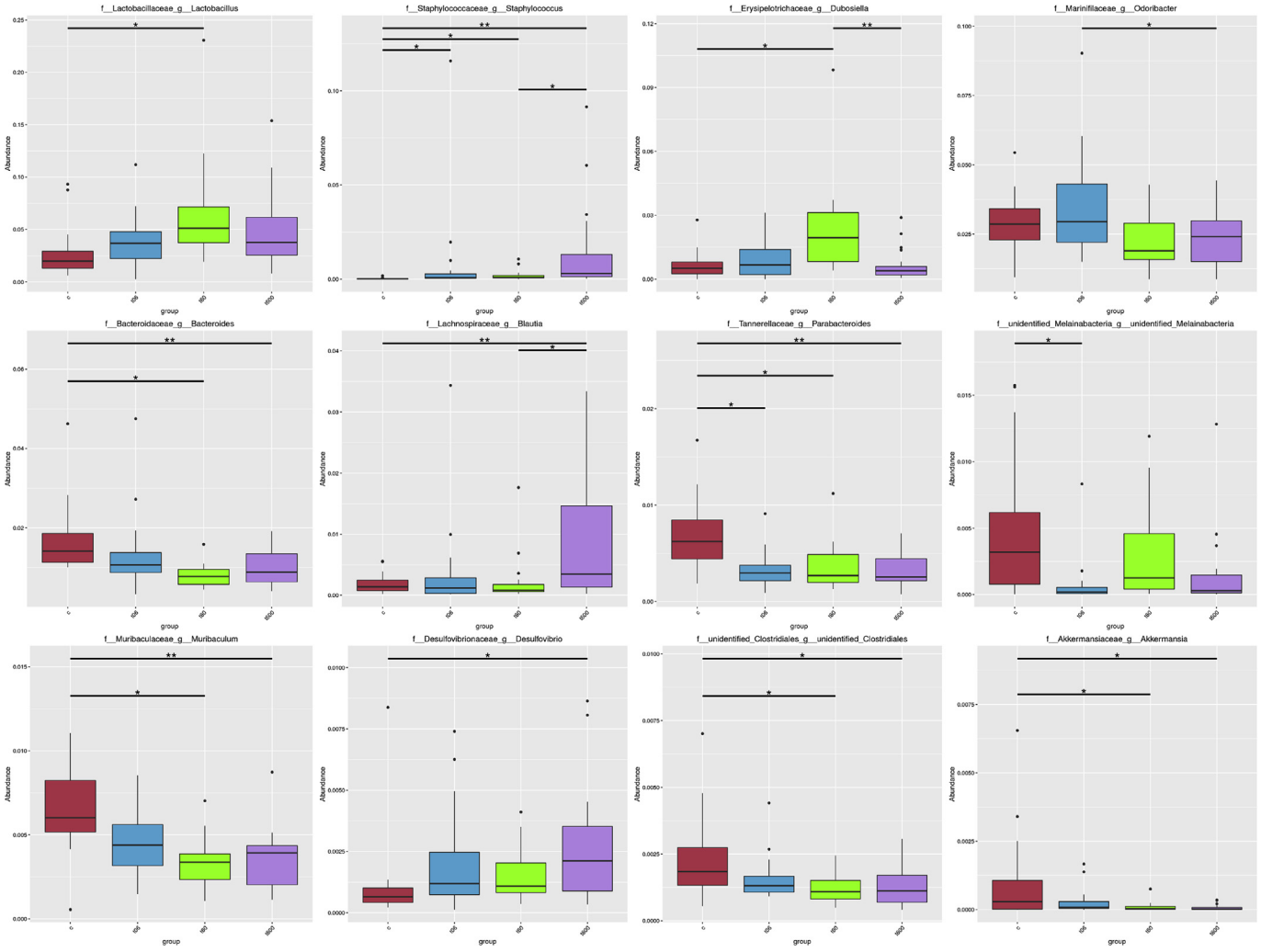


**Fig. 2.** Boxplot showing the distribution of the top 12 genera with different abundances in the c, t06, t60, and t600 groups. Between-group differences were assessed using the MetaStat method. $^{*}P < 0.05$, $^{**}P < 0.01$.

change such as *Staphylococcus* and *Parabacteroides* after feeding mice various concentrations of microplastics for 5 weeks. Compared with the blank group, the feeding groups showed a significant increase in *Staphylococcus* abundance, alongside a significant decrease in *Parabacteroides* abundance ($P < 0.05$). The abundances of *Bacterodides*, *Muribaculum*, *unidentified_Clostridiales*, and *Akkermansia* in the t60 and t600 groups were significantly lower than that in the c group ($P < 0.05$), while these genera in the t06 group showed no obvious difference compared to the c group. The abundance of *unidentified_Melainabacteria* in the t06 group was significantly lower than that in the c group ($P < 0.05$), while its abundance in the t60 and t600 groups showed no obvious difference compared to the c group. The abundance of *Lactobacillus* and *Dubosiella* in the t60 group was significantly higher than that in the c group ($P < 0.05$), while their abundances in the t06 and t600 groups were similar to that in the c group. The abundance of *Blautia* and *Desulfovibrio* in the t600 group was significantly greater than that in the c group ($P < 0.05$), while their abundances in the t06 and t60 groups were not significantly different.

### 3.2. Effects of PE microplastics exposure on cytokine secretion

The concentrations of eight cytokines are summarized in Fig. 3. ANOVA analysis results showed considerable variability between the four groups with respect to the serum concentrations of cytokines (IL-1α, G-CSF, IL-2, IL-5, IL-6, IL-9, IP-10, and RANTES). IL-1α concentrations in the c, t06, t60 and t600 groups were 985.75 ± 359.83, 2232.11 ± 1249.05, 2184.93 ± 1014.97, and 2617.00 ± 1473.13 pg $mL^{-1}$, respectively. The IL-1α concentration in the c group was significantly lower than those in the other three groups ($P < 0.01$). The G-CSF concentration in the c group (1182.90 ± 403.16 pg $mL^{-1}$) was significantly higher than those in the t60 group (737.24 ± 248.96 pg $mL^{-1}$) and t600 group (999.78 ± 279.32 pg $mL^{-1}$) ($P < 0.01$). Compared with the c group, the concentrations of IL-2 and IL-6 in the t06 group were decreased 0.48-fold and increased 3.20-fold, respectively ($P < 0.01$). IP-10 and RANTES concentrations in the t60 group were significantly lower (0.71-fold) and higher (1.38-fold) than that in the c group ($P < 0.01$), respectively. Compared with the c group, IL-5 and IL-9 concentrations were decreased 0.51-fold and increased 1.68-fold in the t600 group.

### 3.3. Effects of PE microplastics exposure on Th17/Treg phenotyping

The percentage of Th17 and Treg cells among $CD4^{+}$ cells and the Th17/Treg ratio were examined in mice spleen cells using the

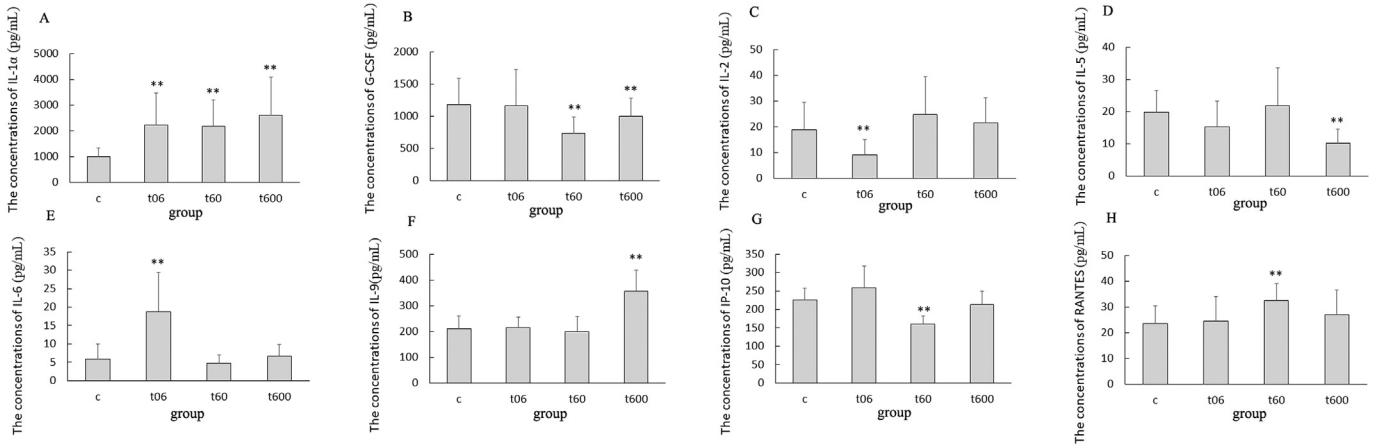


**Fig. 3.** IL-1α (A), G-CSF (B), IL-2 (C), IL-5 (D), IL-6 (E), IL-9 (F), IP-10 (G), and RANTES (H) levels in the c, t06, t60, and t600 groups (mean ± standard deviation [SD]; n = 20, $^{**}P < 0.01$).

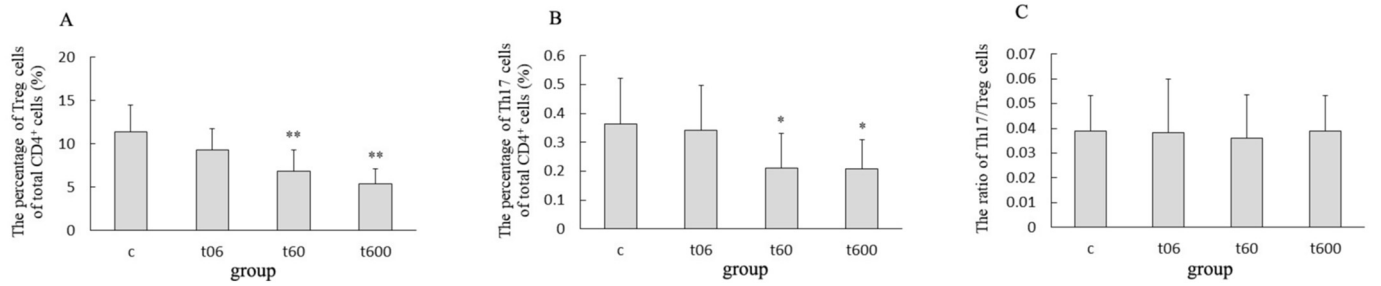


**Fig. 4.** Boxplot representing the levels of Treg, Th17, and Th17/Treg ratio in the c, t06, t60, and t600 groups (mean ± SD; n = 20, $^{*}P < 0.05$ and $^{**}P < 0.01$).

mouse Th17/Treg phenotyping kit (Fig. 4). The results showed a decrease in the percentage of Th17 and Treg cells among $CD4^+$ cells following treatment with 60 μg and 600 μg of microplastics. The percentages of Treg cells among $CD4^+$ cells were 11.41 ± 3.09%, 9.32 ± 2.43%, 6.82 ± 2.50%, and 5.40 ± 1.71% in the c, t06, t60 and t600 groups, respectively. The percentages of Th17 cells among $CD4^+$ cells were 0.36 ± 0.16%, 0.34 ± 0.16%, 0.21 ± 0.12%, and 0.20 ± 0.10% in the c, t06, t60 and t600 groups, respectively. There were no significant differences between the c group and the treatment groups with respect to the Th17/Treg cell ratio.

### 3.4. Effect of PE microplastics on intestinal inflammation of mice

The histological scores of colon and duodenum between the c and t600 group were significantly different (Table S1). Compared to the c group, the colon and duodenum of mice treated with 600 μg of microplastics showed inflammation. In the t600 group, the colon tissue glands were loose; edema and lymphocyte and plasma cell infiltration could be seen in the lamina propria. The duodenum of the t600 group was loose in the glands, with proliferation of small vessels and infiltration of chronic inflammatory cells such as lymphocytes and plasma cells in the lamina propria (Fig. S3).

### 3.5. TLR4, AP-1, and IRF5 expression in the colon and duodenum of PE microplastics-fed mice

TLR4, AP-1, and IRF5 expression in the t600 group was significantly increased compared to that in the c group. In the 600 μg group, the mean fluorescence intensity of TLR4 in the mouse colon was 97.99 ± 24.46, which was significantly higher ($P < 0.01$) than that in the c group (26.21 ± 12.11). Mean fluorescence intensity of TLR4 in the c group was 22.91 ± 6.34, which increased to 44.97 ± 9.78 ($P < 0.01$) in duodenum tissues of mice following ingestion of 600 μg of microplastics (Fig. 5). Following treatment with 600 μg of microplastics, mean fluorescence intensity of AP-1 in the mouse colon was 56.94 ± 13.74, which was higher ($P < 0.01$) than that of the c group (13.84 ± 4.31). Mean fluorescence intensity of AP-1 in the mouse duodenum of the t600 group was 57.42 ± 29.03, which was 1.94-fold higher than that in the c group ($P < 0.05$) (Fig. 6). Following treatment with 600 μg of microplastics, mean fluorescence intensity of IRF7 in the mouse colon was 51.74 ± 19.33, whereas mean fluorescence intensity of IRF7 in the c group was lower ($P < 0.01$), with mean ± standard deviation values of 12.45 ± 7.23. The mean fluorescence intensity of IRF7 in the t600 group was significantly higher (2.51 times) than that in the c group ($P < 0.01$) (Fig. 7). There were no significant differences among the c, t06 and t60 groups with respect to TLR4, AP-1, and IRF5 expression in mice colon and duodenum tissues (data not shown).

## 4. Discussion

Microplastics are a new pollutant with persistence, bioaccumulation, and toxicity, and their impact on human health is particularly concerning. Microplastics can enter the human body through the food chain (web), consuming sea salt and drinking water (Setälä et al., 2014; Yang et al., 2015; Kosuth et al., 2018). Food web transfer experiments have shown that mysid shrimps, copepods, Cladocera, rotifers, polychaete larvae, and ciliates could ingest 10 μm-sized microspheres (Setälä et al., 2014). Approximately 55% of the microplastics in table salt from China measured less than 200 μm in size (Yang et al., 2015). The vast majority

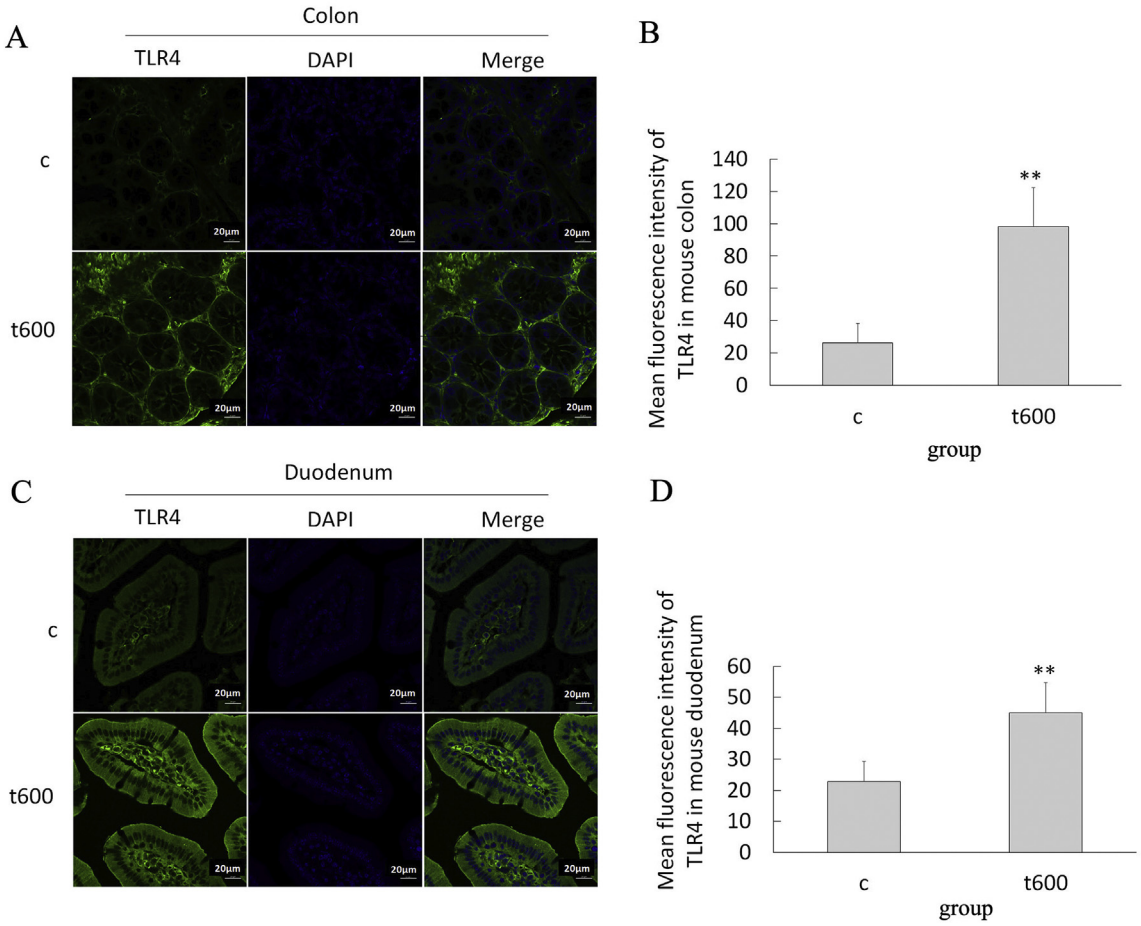


**Fig. 5.** TLR4 expression in the colon and duodenum in the c and t600 groups. (A) Immunostaining of TLR4 in colon tissues of the c and t600 groups. Green indicates TLR4 staining. Nuclei (blue) were stained with DAPI. Scale bar: 20 μm. (B) Fluorescence intensity of TLR4 in colon tissues of the c and t600 groups. $^{**}P < 0.01$. (C) Immunostaining of TLR4 in duodenum tissues of the c and t600 groups. Green indicates TLR4 staining. Nuclei (blue) were stained with DAPI. Scale bar: 20 μm. (D) Fluorescence intensity of TLR4 in duodenum tissues of the c and t600 groups. $^{**}P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(98.4%) of microplastics in tap water are classified as fibers with a length range of 0.1–5 mm (Kosuth et al., 2018). Organisms at every level of the food web likely ingest microplastics, although the speculated quantities ($\mu g \cdot g^{-1}$; $ng \cdot g^{-1}$) of microplastics vary for each biological species (Ivar do Sul and Costa, 2014).

The fecal microbiota composition with a combination of shed mucosal bacteria and a separate non-adherent luminal population can reflect gut microflora in mice (Li et al., 2017; Eckburg et al., 2005). In this study, mice fed high concentrations of PE microplastics showed a significant increase in the numbers of gut microbes, bacterial abundance, and flora diversity as compared to that in the blank group (Fig. 1). The extent of change in bacterial diversity showed a progressive increase with increasing microplastics concentration (Scherer et al., 2017). PS microplastics have been shown to alter the intestinal microbiota composition and thereby induce dysbiosis (Lu et al., 2018; Jin et al., 2019). Gut flora has special physiological functions due to its diversity. Normally, gut flora is in a relatively stable equilibrium state. In this study, the balance of gut microflora was altered following ingestion of a high concentration of microplastics, which might lead to the occurrence of related diseases.

Gut microbiota is dominated by bacteria, and more than 90% of the species belong to Bacteroidetes and Firmicutes. Bacteroidetes decreased, and Firmicutes and Melainabacteria phyla increased in gut microbiota of mice following treatment with PE microplastics (Fig. S2). Previous studies have shown that overgrowth of *Staphylococcus* is associated with inflammatory bowel disease due to its superantigen-induced inflammation (Collado et al., 2008). As shown in Fig. 2, the feeding groups showed a significant increase in *Staphylococcus* genus abundance, alongside a significant decrease in *Parabacteroides* genus abundance ($P < 0.05$). Noor et al. (2010) found that expression levels of *Parabacteroides* in patients with ulcerative colitis and irritable bowel syndrome were significantly lower than that of normal individuals. Therefore, PE microplastics-induced changes in the genera of *Staphylococcus* and *Parabacteroides* might cause intestinal inflammation and other related diseases. The abundances of *Bacteroides*, *Muribaculum*, unidentified_Clostridiales, and *Akkermansia* genera in the t60 and t600 groups were significantly lower than that in the c group ($P < 0.05$). *Bacteroides* and *Muribaculum* genera maintain the ecological balance of microflora (Liu et al., 2018). The order Clostridiales contributed strongly to the production of short-chain fatty acids in the gut (Ferrario et al., 2014), which play an important role in maintaining the metabolism of fatty acids, sugar, and cholesterol (Setälä et al., 2014). Individuals with fewer *Akkermansia* in the intestine were more likely to gain weight, exhibit disrupted intestinal barrier function, and develop inflammation (Kang et al., 2013). As shown in Fig. 2, the abundances of *Lactobacillus* and *Dubosiella* in the t60 group were significantly higher than that in the c group ($P < 0.05$), and the abundances of *Blautia* and *Desulfovibrio* in the

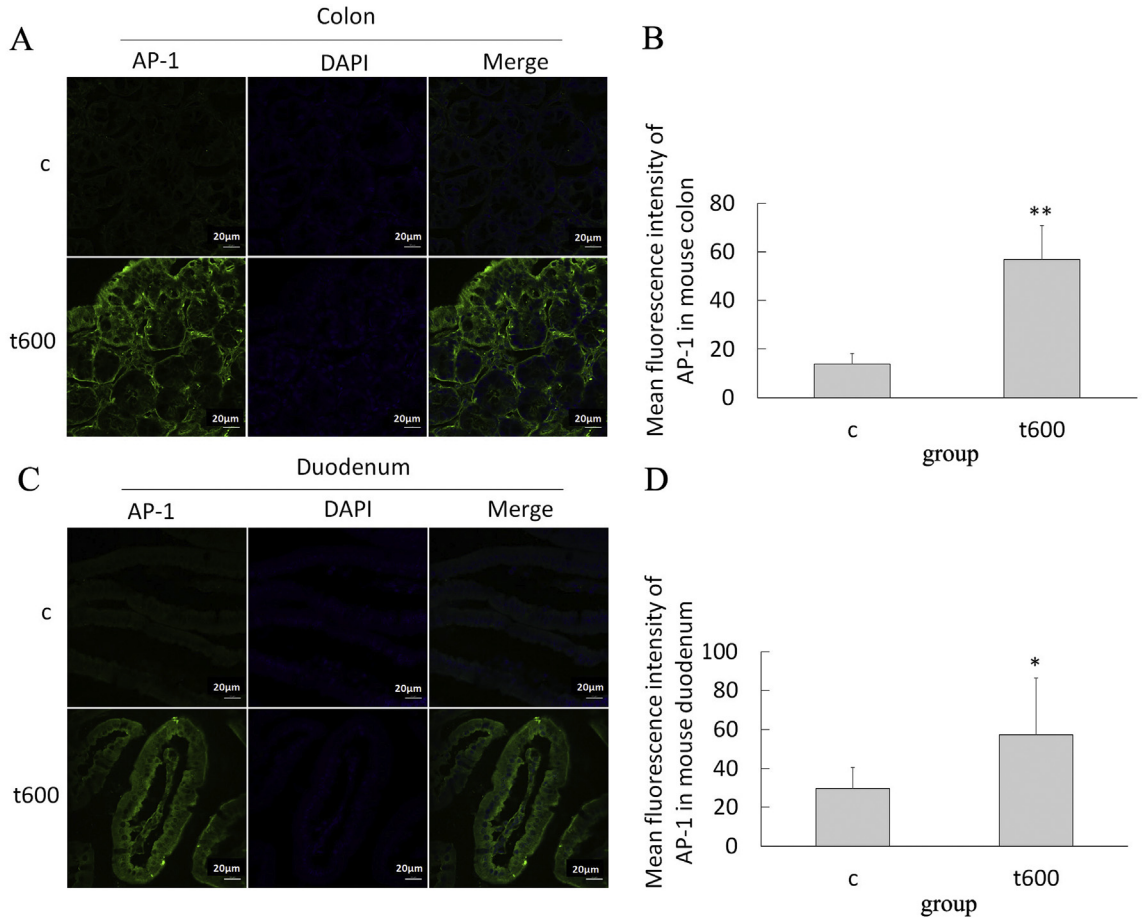


**Fig. 6.** AP-1 expression in the colon and duodenum tissues in the c and t600 groups. (A) Immunostaining of AP-1 in colon tissues of the c and t600 groups. Green indicates AP-1 staining. Nuclei (blue) were stained with DAPI. Scale bar: 20 μm. (B) Fluorescence intensity of AP-1 in colon tissues of the c and t600 groups. $^{**}P < 0.01$. (C) Immunostaining of AP-1 in duodenum tissues of the c and t600 groups. Green indicates AP-1 staining. Nuclei (blue) were stained with DAPI. Scale bar: 20 μm. (D) Fluorescence intensity of AP-1 in duodenum tissues of the c and t600 groups. $^{*}P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

t600 group showed similar trends. *Lactobacillus*, an intestinal probiotic, has been shown to enhance both systemic and mucosal immunity (Galdeano and Perdigón, 2006). *Blautia* is a known anti-inflammatory molecule that can produce butyrate (Tyler et al., 2013); *Desulfovibrio* also contributes to the inflammatory process, leading to metabolic disease (Zhang-Sun et al., 2015). Altogether, PE microplastics had a profound impact on the composition and diversity of gut microflora in mice, and this disturbance could break the balance of intestinal micro-ecology and induce intestinal inflammation.

Gut microbiota can influence innate immunity and adaptive immunity through their own components or metabolites, and produce effector molecules and the immune response (Arnolds and Lozupone, 2016). Here, we detected serum levels of cytokines (IL-1α, G-CSF, IL-2, IL-5, IL-6, IL-9, IP-10, and RANTES) (Fig. 3) and the percentages of Th17 and Treg cells among $CD4^{+}$ cells in splenocytes of mice following ingestion of different amounts of PE microplastics (Fig. 4). As shown in Fig. 3, the concentration of IL-1α in the c group was significantly lower than in the PE microplastics-fed groups ($P < 0.01$). A previous study demonstrated that *Staphylococcus aureus* infection led to expression of the pro-inflammatory cytokine IL-1α in a brain abscess model (Kielian et al., 2004). Therefore, PE microplastics treatment could increase abundace of *Staphylococcus* genera in gut microbiota, and upregulation of *Staphylococcus* abundance might induce the increase in IL-1α. G-CSF can absorb neutrophils from the blood into inflammatory tissues and play an important role in chronic inflammation (Wengner et al., 2008). G-CSF has a cytoprotective effect on various stresses of neuronal and cardiac cells (Meshkibaf et al., 2018). The expression of G-CSF decreased following ingestion of higher concentrations of PE microplastics, indicating that the immune protective effect of G-CSF was weakened. Compared with the c group, the concentrations of IL-2 and IL-6 in the t06 group were decreased and increased, respectively ($P < 0.01$). IL-2 plays an important role not only in the development and expansion of effector T cells, but also in the establishment and maintenance of immune tolerance (Zorn et al., 2006). The pro-inflammatory cytokine IL-6 is a key regulator of sporadic and inflammatory bowel disease (Kasza, 2013). The decrease in IL-2 in the t06 group has been implicated in reduction of the incidence of intestinal inflammatory diseases. The concentrations of IP-10 and RANTES in the t60 group were significantly lower (0.71-fold) and higher (1.38-fold) than those in the c group ($P < 0.01$), respectively. RANTES not only plays an important role in pulmonary allergic inflammation, pulmonary leukocyte infiltration, and eosinophil recruitment, but also plays a role in the neuro-inflammatory response (Conti and DiGioacchino, 2001). As a pro-inflammatory mediator, IP-10 can activate and attract phagocytic cells and Th1 immune cells to the site of infection (Hoermannsperger et al., 2009). The decrease in IP-10 was beneficial in the alleviation of intestinal inflammation in mice of the t60 group. Compared with the c group, IL-5 and IL-9 concentrations were decreased 0.51-fold and increased 1.68-fold in the t600 group,

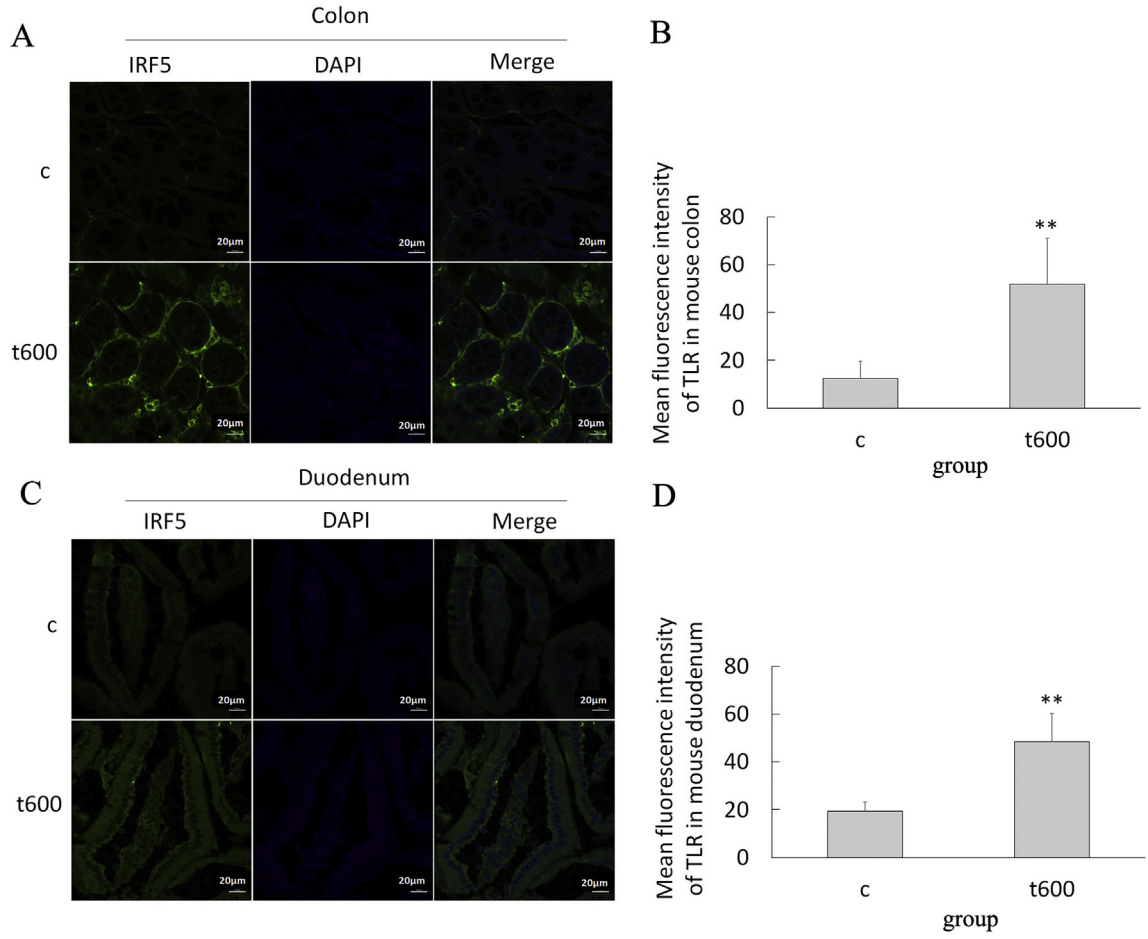


**Fig. 7.** IRF5 expression in colon and duodenum tissues in the c and t600 groups. (A) Immunostaining of IRF5 in colon tissues of the c and t600 groups. Green indicates IRF5 staining. Nuclei (blue) were stained with DAPI. Scale bar: 20 μm. (B) Fluorescence intensity of IRF5 in colon tissues of the c and t600 groups. $^{**}P < 0.01$. (C) Immunostaining of IRF5 in duodenum tissues of the c and t600 groups. Green indicates IRF5 staining. Nuclei (blue) were stained with DAPI. Scale bar: 20 μm. (D) Fluorescence intensity of IRF5 in duodenum tissues of the c and t600 groups. $^{**}P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

respectively. IL-5 maintains the functional activity of mature eosinophils and induces the production of superoxide anions (Yamaguchi et al., 1988). IL-9 is not only involved in the recruitment of mast cells and the regulation of effector function, but is also a multipotent cytokine of the Th2 inflammatory response (Forbes et al., 2008). Therefore, PE microplastics may affect the inflammatory response via separate pathways, and ingestion of a high concentration of PE microplastics tended to result in the development of inflammation.

Cytokines play an important role in determining the differentiation of naïve T cells to Th17 or Treg cells (Omenetti and Pizarro, 2015). Inflammatory response and cytokine production have been shown to be dependent on intestinal bacteria (Goto et al., 2014). Our results showed that intake of PE microplastics might potentially reduce the percentage of Th17 and Treg among $CD4^+$ cells; however, no significant differences were observed among groups with respect to the Th17/Treg cell ratio. These results are consistent with previous studies that showed a regulatory effect of gut microbiota on Treg/Th17 cells. In germ-free mice, the frequencies of Th17 and Treg cells were decreased (Atarashi et al., 2011; Ivanov et al., 2008). *B. fragilis* in the intestinal tract was shown to promote the conversion of $CD4^+$ T cells to Treg cells and inhibit the expansion of Th17 cells (Round et al., 2011). Induction of Treg formation by *Clostridium* was shown to be crucial for gut immune homeostasis (Atarashi et al., 2013). Therefore, PE microplastics may regulate the numbers of Th17 and Treg cells by altering gut microbiota diversity.

Gut microflora can activate and regulate signal transduction pathways related to intestinal mucosal immune function, such as the TLR pathway, and form intestinal defense mechanisms to jointly resist the invasion of foreign substances (Kamada et al., 2013). Histological scores of the colon and duodenum of the c and t600 groups were significantly different (Table S1). In the t600 group, the colon tissue glands were loose, and edema and lymphocyte and plasma cell infiltration could be seen in the lamina propria. The duodenum of the t600 group was loose in glands, with proliferation of small vessels and infiltration of chronic inflammatory cells such as lymphocytes and plasma cells in the lamina propria (Fig. S3). These data indicated that the intestine (colon and duodenum) of mice fed 600 μg of microplastics was inflamed. TLRs appear to be key regulators that induce inflammatory activation of the mucosal immune response (Cario and Podolsky, 2000); TLR4 protein levels have been shown to be elevated in inflammation-dependent colonic mucosa of children with irritable bowel syndrome (Szebeni et al., 2008). AP-1 and IRF5 are both pro-inflammatory transcription factors downstream of TLR4 (Jung et al., 2009; Weiss et al., 2015). In this study, TLR4, AP-1, and IRF5 expression in the t600 group was significantly increased compared to that in the c group (Figs. 5–7), indicating that activation of TLR4/AP-1 and TLR4/IRF5 signaling is important in the intestinal

inflammation of mice fed 600 μg of microplastics. Our results may facilitate a better understanding of the mechanism by which microplastics induce intestinal dysbacteriosis and inflammation.

## 5. Conclusion

The composition and diversity of intestinal microflora were altered in mice following ingestion of PE microplastics, and gut microbiota in the group fed a high concentration of microplastics showed more microbial species and increased bacterial abundance and flora diversity. Different amounts of microplastics could increase the secretion of pro-inflammatory cytokine IL-1α in serum, but each amount of microplastics affected the secretion of specific cytokines. Microplastics decreased the percentage of Th17 and Treg cells among $CD4^+$ cells, but the Th17/Treg cell ratio remained unchanged. High-concentration microplastics tended to induce intestinal inflammation through activation of TLR4 signaling. PE microplastics can induce intestinal dysbacteriosis and inflammation, providing a theoretical basis for the prevention and treatment of microplastics-related diseases.

## Funding

This work was supported by grants from the National Natural Science Foundation of China (grant numbers: 81771709 and 81471561); and the Youth Prominent Teacher Training Plan of Binzhou Medical University; Department of Education of Shandong Province (2019KJK012).

## Ethics approval

All mouse experimental procedures were approved by the ethics committees of Binzhou Medical University, which complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

## Declaration of competing interest

The authors declare that they have no competing interests.

## Acknowledgments

We are grateful to Professor Haixia Zhang of Binzhou Medical University for her guidance on the experimental techniques in this study.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.125492.

## References

Arnolds, K.L., Lozupone, C.A., 2016. Striking a balance with help from our little friends – how the gut microbiota contributes to immune homeostasis. Yale J. Biol. Med. 89, 389–395.
Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., et al., 2011. Induction of colonic regulatory T cells by indigenous Clostridium species. Science 331, 337–341.
Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., et al., 2013. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature 500, 232–236.
Brannock, P.M., Halanych, K.M., 2015. Meiofaunal community analysis by high-throughput sequencing: comparison of extraction, quality filtering, and clustering methods. Mar. Genomics 23, 67–75.
Cario, E., Podolsky, D.K., 2000. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect. Immun. 68, 7010–7017.
Collado, M.C., Isolauri, E., Laitinen, K., Salminen, S., 2008. Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. Am. J. Clin. Nutr. 88, 894–899.
Conti, P., DiGioacchino, M., 2001. MCP-1 and RANTES are mediators of acute and chronic inflammation. Allergy Asthma Proc. 22, 133–137.
Costello, E.K., Lauber, C.L., Hamady, M., Fierer, N., Gordon, J.I., Knight, R., 2009. Bacterial community variation in human body habitats across space and time. Science 326, 1694–1697.
Cui, H., Cai, Y., Wang, L., Jia, B., Li, J., Zhao, S., et al., 2018. Berberine regulates Treg/Th17 balance to treat ulcerative colitis through modulating the gut microbiota in the colon. Front. Pharmacol. 9, 571.
Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., et al., 2005. Diversity of the human intestinal microbial flora. Science 308, 1635–1638.
El Aidy, S., Van Baarlen, P., Derrien, M., Lindenbergh-Kortleve, D.J., Hooiveld, G., Levenez, F., et al., 2012. Temporal and spatial interplay of microbiota and intestinal mucosa drive establishment of immune homeostasis in conventionalized mice. Mucosal Immunol. 5, 567–579.
Ellis, A.R., Burchett, W.W., Harrar, S.W., Bathke, A.C., 2017. Nonparametric inference for multivariate data: the R package npmv. J. Stat. Softw. 76, 1–18.
Ferrario, C., Taverniti, V., Milani, C., Fiore, W., Laureati, M., De Noni, I., et al., 2014. Modulation of fecal Clostridiales bacteria and butyrate by probiotic intervention with Lactobacillus paracasei DG varies among healthy adults. J. Nutr. 144, 1787–1796.
Forbes, E.E., Groschwitz, K., Abonia, J.P., Brandt, E.B., Cohen, E., Blanchard, C., et al., 2008. IL-9- and mast cell-mediated intestinal permeability predisposes to oral antigen hypersensitivity. J. Exp. Med. 205, 897–913.
Frank, D.N., St Amand, A.L., Feldman, R.A., Boedeker, E.C., Harpaz, N., Pace, N.R., 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel disease. Proc. Natl. Acad. Sci. U. S. A. 104, 13780–13785.
Galdeano, C.M., Perdigón, G., 2006. The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. Clin. Vaccine Immunol. 3, 219–226.
Galloway, T.S., Lewis, C.N., 2016. Marine microplastics spell big problems for future generations. Proc. Natl. Acad. Sci. U. S. A. 113, 2331–2333.
Goto, Y., Panea, C., Nakato, G., Cebula, A., Lee, C., Diez, M.G., et al., 2014. Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation. Immunity 40, 594–607.
Hoermannsperger, G., Clavel, T., Hoffmann, M., Reiff, C., Kelly, D., Loh, G., et al., 2009. Post-translational inhibition of IP-10 secretion in IEC by probiotic bacteria: impact on chronic inflammation. PLoS One 4, e4365.
Ivanov, I.I., Frutos Rde, L., Manel, N., Yoshinaga, K., Rifkin, D.B., Sartor, R.B., et al., 2008. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe 4, 337–349.
Ivar do Sul, J.A., Costa, M.F., 2014. The present and future of microplastic pollution in the marine environment. Environ. Pollut. 185, 352–364.
Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., et al., 2015. Marine pollution. Plastic waste inputs from land into the ocean. Science 347, 768–771.
Jin, Y., Lu, L., Tu, W., Luo, T., Fu, Z., 2019. Impacts of polystyrene microplastic on the gut barrier, microbiota and metabolism of mice. Sci. Total Environ. 649, 308–317.
Jung, K.J., Lee, E.K., Kim, J.Y., Zou, Y., Sung, B., Heo, H.S., et al., 2009. Effect of short term calorie restriction on pro-inflammatory NF-kB and AP-1 in aged rat kidney. Inflamm. Res. 58, 143–150.
Kamada, N., Seo, S.U., Chen, G.Y., Núñez, G., 2013b. Role of the gut microbiota in immunity and inflammatory disease. Nat. Rev. Immunol. 13, 321–335.
Kang, C.S., Ban, M., Choi, E.J., Moon, H.G., Jeon, J.S., Kim, D.K., et al., 2013. Extracellular vesicles derived from gut microbiota, especially Akkermansia muciniphila, protect the progression of dextran sulfate sodium-induced colitis. PLoS One 8, e76520.
Kasza, A., 2013. IL-1 and EGF regulate expression of genes important in inflammation and cancer. Cytokine 62, 22–33.
Kielian, T., Bearden, E.D., Baldwin, A.C., Esen, N., 2004. IL-1 and TNF-alpha play a pivotal role in the host immune response in a mouse model of *Staphylococcus aureus*-induced experimental brain abscess. J. Neuropathol. Exp. Neurol. 63, 381–396.
Kosuth, M., Mason, S.A., Wattenberg, E.V., 2018. Anthropogenic contamination of tap water, beer, and sea salt. PLoS One 13, e0194970.
Li, S., Wang, Z., Yang, Y., Yang, S., Yao, C., Liu, K., et al., 2017. *Lachnospiraceae* shift in the microbial community of mice faecal sample effects on water immersion restraint stress. Amb. Express 7, 82.
Liao, M., Xie, Y., Mao, Y., Lu, Z., Tan, A., Wu, C., et al., 2018. Comparative analyses of fecal microbiota in Chinese isolated Yao population, minority Zhuang and rural Han by 16sRNA sequencing. Sci. Rep. 8, 1142.
Liu, C., Liu, L., Zhou, Q., Xin, Y., Zhang, B., Zhang, L., et al., 2018. Effects of functional food on blood glucose and intestinal microbiota in type 2 diabetes mice. Chin. J. Microecol. 30, 781–784.
Lu, L., Wan, Z., Luo, T., Fu, Z., Jin, Y., 2018. Polystyrene microplastics induce gut microbiota dysbiosis and hepatic lipid metabolism disorder in mice. Sci. Total Environ. 631–632, 449–458.
Luo, Y., Zhou, Q., Zhang, H., Pan, X., Tu, C., Li, L., et al., 2018. Pay Attention to Research on Microplastic Pollution in Soil for Prevention of Ecological and Food Chain Risks, vol. 33. China Academic Journal Electronic Publishing House,

pp. 1021–1030.
Meshkibaf, S., Fritz, J., Gottschalk, M., Kim, S.O., 2018. Preferential production of G-CSF by a protein-like Lactobacillus rhamnosus GR-1 secretory factor through activating TLR2-dependent signaling events without activation of JNKs. BMC Microbiol. 15, 238.
Nadler, E.P., Dickinson, E., Knisely, A., Zhang, X.R., Boyle, P., Beer-Stolz, D., et al., 2000. Expression of inducible nitric oxide synthase and interleukin-12 in experimental necrotizing enterocolitis. J. Surg. Res. 92, 71–77.
Noor, S.O., Ridgway, K., Scovell, L., Kemsley, E.K., Lund, E.K., Jamieson, C., et al., 2010. Ulcerative colitis and irritable bowel patients exhibit distinct abnormalities of the gut microbiota. BMC Gastroenterol. 10, 134.
Obrenovich, M.E., Tima, M., Polinkovsky, A., Zhang, R., Emancipator, S.N., Donskey, C.J., 2017. Targeted metabolomics analysis identifies intestinal microbiota-derived urinary biomarkers of colonization resistance in antibiotic-treated mice. Antimicrob. Agents Chemother. 61 e00477-17.
Omenetti, S., Pizarro, T.T., 2015. The Treg/Th17 Axis: a dynamic balance regulated by the gut microbiome. Front. Immunol. 6, 639.
Round, J.L., Lee, S.M., Li, J., Tran, G., Jabri, B., Chatila, T.A., et al., 2011. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. Science 332, 974–977.
Scherer, C., Brennholt, N., Reifferscheid, G., Wagner, M., 2017. Feeding type and development drive the ingestion of microplastics by freshwater invertebrates. Sci. Rep. 7, 17006.
Schirmer, M., Smeekens, S.P., Vlamakis, H., Jaeger, M., Oosting, M., Franzosa, E.A., et al., 2016. Linking the human gut microbiome to inflammatory cytokine production capacity. Cell 167, 1125–1136.
Setälä, O., Fleming-Lehtinen, V., Lehtiniemi, M., 2014. Ingestion and transfer of microplastics in the planktonic food web. Environ. Pollut. 185, 77–83.
Szebeni, B., Veres, G., Dezsõfi, A., Rusai, K., Vannay, A., Mraz, M., et al., 2008. Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease. Clin. Exp. Immunol. 151, 34–41.
Teuten, E.L., Saquing, J.M., Knappe, D.R., Barlaz, M.A., Jonsson, S., Björn, A., et al., 2009. Transport and release of chemicals from plastics to the environment and to wildlife. Philos. Trans. R. Soc. Lond. B Biol. Sci. 364, 2027–2045.
Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W., et al., 2004. Lost at sea: where is all the plastic? Science 304, 838.
Tyler, A.D., Knox, N., Kabakchiev, B., Milgrom, R., Kirsch, R., Cohen, Z., et al., 2013. Characterization of the gut-associated microbiome in inflammatory pouch complications following ileal pouch-anal anastomosis. PLoS One 8, e66934.
Underwood, M.A., 2014. Intestinal dysbiosis: novel mechanisms by which gut microbes trigger and prevent disease. Prev. Med. 65, 133–137.
Weiss, M., Byrne, A.J., Blazek, K., Saliba, D.G., Pease, J.E., Perocheau, D., et al., 2015. IRF5 controls both acute and chronic inflammation. Proc. Natl. Acad. Sci. U. S. A. 112, 11001–11006.
Wengner, A.M., Pitchford, S.C., Furze, R.C., Rankin, S.M., 2008. The coordinated action of G-CSF and ELR + CXC chemokines in neutrophil mobilization during acute inflammation. Blood 111, 42–49.
Wu, C., Pan, X., Shi, H., Peng, J., 2018. Microplastic Pollution in Freshwater Environment in China and Watershed Management Strategy, vol. 33. China Academic Journal Electronic Publishing House, pp. 1012–1020.
Yamaguchi, Y., Hayashi, Y., Sugama, Y., Miura, Y., Kasahara, T., Kitamura, S., et al., 1988. Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs in vitro survival. IL-5 as an eosinophil chemotactic factor. J. Exp. Med. 167, 1737–1742.
Yang, D., Shi, H., Li, L., Li, J., Jabeen, K., Kolandhasamy, P., 2015. Microplastic pollution in table salts from China. Environ. Sci. Technol. 49, 13622–13627.
Zhang-Sun, W., Augusto, L.A., Zhao, L., Caroff, M., 2015. Desulfovibrio desulfuricans isolates from the gut of a single individual: structural and biological lipid A characterization. FEBS Lett. 589, 165–171.
Zhao, J., Ran, W., Teng, J., Liu, Y., Liu, H., Yin, X., et al., 2018. Microplastic pollution in sediments from the bohai sea and the yellow sea, China. Sci. Total Environ. 640–641, 637–645.
Zorn, E., Nelson, E.A., Mohseni, M., Porcheray, F., Kim, H., Litsa, D., et al., 2006. IL-2 regulates *FOXP3* expression in human $CD4^{+}CD25^{+}$ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. Blood 108, 1571–1579.