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Effects of Microplastics on the Gastrointestinal Tract and Gut Microbiome of Sprague Dawley Rats

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ABSTRACT: Humans are commonly exposed to microplastics (MPs) via food. However, relatively little is known of their effect on the tissue of the gastrointestinal tract and the gut microbiome. In this study, adult male rats were fed with different concentrations (0 to 1000 μg/day) of polyethylene MPs mixed with standard pellet food for 24 days. At the end of the experiment, rats were euthanized and histopathological investigation using light and electron microscopy was performed. The gut microbiome was analyzed using MiSeq 16S rRNA amplicon sequencing. Microscopic analysis revealed a significant impact of MPs on the intestine, including visible changes to the crypt area and reduction in mucus secretion. Autophagic vacuoles were observed in the livers of the rats fed with MPs at 100 µg/day. Oxidative stress was evident in rats fed with 1000 µg/day of MPs, indicated by the presence of myeloid bodies in both intestinal and hepatic cells. The gut microbiome was also affected by MPs. Although no distinct clusters were observed in the different treatments by multidimensional scaling (NMDS). The diversity indices including operational taxonomic unit (OTU) richness and Chao1 exhibited an increasing trend with increasing MPs concentration. Pearson correlations revealed a linear increase in the relative abundance of *Clostridia* ($R = 0.036$, $p = 0.003$) with increasing MPs concentration. *Lactobacillus faecis* was the most abundant OTU in the entire dataset, and its relative abundance decreased significantly with increasing MPs concentration. This study demonstrates that MPs can disrupt cellular function and disturb microbiome balance, and in the event of prolonged exposure, organisms might experience harmful effects.

Keywords: MPs; PET; Microbiome; Myeloid inclusions; Peroxisomes.

تأثير اللدائن الرقيقة من البلاستيك على الجهاز الهضمي وميكروبيوم معدة جرذان سبراجو داولي

زهرة المحروقي، طاهر باعمر، مايكل باري، عزيز الحبسي و رائد عابد

الملخص: تعرضت الحيوانات للدائن الرقيقة من البلاستيك MP عن طريق الطعام، ومع ذلك، لا يوجد الكثير من المعلومات المعروفة عن تأثيرها على أنسجة الجهاز الهضمي وميكروبيوم الأمعاء. في هذه الدراسة، تم تغذية ذكور الجرذان البالغة بتركيزات مختلفة (0 إلى 1000 ميكروغرام / يوم) من البولي إيثيلين الممزوج بأطعمة الحبيبات القياسية لمدة 24 يومًا. وفي نهاية التجربة، تم القتل الرحيم للجرذان وتم إجراء فحص الأنسجة المرضية باستخدام المجهر الضوئي والإلكتروني. وتم تحليل ميكروبيوم الأمعاء باستخدام تسلسل MiSeq 16 rRNA amplicon S. كشف التحليل المجهري عن تأثير كبير للMPs على الأمعاء، بما في ذلك التغيرات المرئية في منطقة القبو وانخفاض إفراز المخاط. وقد لوحظ وجود فجوات ذاتية الالتهام في كبد الجرذان التي تم تغذيتها بـ MPs بمعدل 100 ميكروغرام / يوم. وكان الإجهاد التأكسدي واضحًا في الجرذان التي تم تغذيتها بـ 1000 ميكروغرام / يوم من البروتينات، وهو ما يشير إلى وجود الأجسام النخاعية في كل من الخلايا المعوية والكبدية. كما تأثر الميكروبيوم المعوي أيضًا بالأعضاء البرلمانية. على الرغم من عدم ملاحظة أي مجموعات متميزة في المعالجات المختلفة عن طريق القياس متعدد الأبعاد (NMDS). أظهرت مؤشرات التنوع بما في ذلك ثراء وحدة التصنيف التشغيلية (OTU) وChao1 اتجاهًا متزايدًا مع زيادة تركيز الجسيمات البلاستيكية . ولقد كشفت ارتباطات بيرسون عن زيادة خطية في وفرة التسلسل

النسبي لكلوستريديا (R = 0.036، p = 0.003) مع زيادة تركيز الجسيمات البلاستيكية. لقد كانت Lactobacillus faecis هي OTU الأكثر وفرة في مجموعة البيانات بأكملها، وانخفضت وفرتها النسبية بشكل ملحوظ مع زيادة تركيز اللدائن البلاستيكية. أظهرت هذه الدراسة أن الجسيمات البلاستيكية يمكن أن تعطل الوظيفة الخلوية وتخل بتوازن ميكروبيوم الامعاء، وفي حالة التعرض لفترات طويلة، قد تواجه الكائنات الحية تأثيرات غير متوقعة.

الكلمات المفتاحية: MP، PET، Microbiome، TEM، SEM، شوائب النخاع الشوكي، الجسيمات التأكسدية، الجيوب الأنفية، الخبايا.

1. Introduction

Plastic production has grown exponentially since the 1950s to 367 million tons in 2020 [1]. Polyethylene teraphate (PET) and polystyrene (PS) are among the most commonly manufactured plastics according to the European plastic database of 2017 [2]. The unprecedented expansion of plastic production has led to widespread plastic pollution in marine as well as terrestrial ecosystems [3]. The macroplastics are easy to identify and have been shown to cause acute threats to marine wildlife and birds. The widespread occurrence of MPs and their potential risks to animals and humans have only recently been identified [4,5]. MPs can be categorized as either primary and secondary [6]. Primary MPs are manufactured for cosmetics, mining, facial cleansers, sunscreens, detergents, and as drug vectors [1,7], whereas secondary MPs are formed through the effects of physical stress (including photo-, bio-, and ultraviolet radiation) that degrade larger polymers into small fragments [8,9]. There is now abundant evidence that MPs can be transferred from one trophic level to the next through the food web [10,11,12]. Previous studies have shown that MPs (including PP and PET) were found in canned sardines and 55% of table salt samples in China were contaminated with MPs [13].

The MPs are generally ingested by animals [14,15], and there is a high possibility that the ingested MPs would cause physical and chemical damage to animal tissues [16]. MPs translocate by circulation to different parts of animal bodies, and eventually accumulate in tissues [17,18,19]. MPs are more likely to translocate to lymphatic and circulatory tissues because of their size. For instance, M-cells and dendritic cells that line the mucosa allow MPs to enter the bloodstream and lymphatic system and accumulate in secondary organs such as the liver [18,20]. Consequently, MPs can trigger immune responses and disrupt cellular structure [17]. MPs can also induce radical oxidative stress (ROS), which can activate signalling pathways, induce inflammation, and promote apoptosis [21].

Several studies have shown that MPs may also alter the gut microbiota of animals and humans [20,21,22]. For instance, the dominant bacterial taxa in mouse feces changed from Bacteroidetes and Firmicutes to Proteobacteria when mice were fed with food containing PET-MPs [23]. Exposure of adult zebrafish to 1000 µg/day of MPs with 0.5 µm and 50 µm diameters increased the abundance of Proteobacteria in the gut microbiome [24]. Proteobacteria are considered as an indicator of intestinal inflammation. MPs consumption has also been linked to increases in the dominance of *Staphylococcus* in the intestinal flora, which is associated with inflammatory bowel disease [18]. *Staphylococcus* can trigger inflammation through superantigens [23]. In general, beneficial bacteria in the gut could be reduced by oxidative stress, allowing opportunistic bacteria to colonize [22]. This study aimed to examine the effects of different concentrations of MPs (0, 5, 50, 100, 500, and 100 µg/day) on the key organs in rats. The histopathological structure and morphology of the rat tissues were investigated using light and electron microscopy. The impact of MPs on the gut microbiome composition was also studied using MiSq 16S rRNA amplicon sequencing.

2. Materials and methods

2.1. Preparation and characterization of MPs

Naturally aged PET water bottles were collected from a beach in northern Oman (N: 23.7075637, E: 57.9331499). They were cut into small pieces and crushed using a steel grinder (Mixer Grinder, Panasonic, MX-AC210s, India). The particles were sliced twice, through a sheaf of 100 μm and a sheaf of 80 μm. The fraction below 80 μm was selected and a granulometry analysis was performed with Fourier-transform infrared spectroscopy (FTIR) to determine the chemical composition of the plastic. The MPs were then examined under a Field Emission Scanning Electron Microscope (FE-SEM, JSM-7600F Schottky, JEOL).

2.2. Rat exposure to different concentrations of MPs

Twenty-four, healthy six-week-old, male Sprague Dawley rats with an average weight of 152±3 g were housed individually in standard cages at 21±1ºC and humidity of 40-60% in a small animal house at SQU. The rats were maintained with a 12h light/dark cycle and were provided with deionized water *ad libitum* and fed with a standard rat food (Barely, Oman flour Mills Company, SAOG, Oman). The rats were acclimatized for one week before being randomly assigned to six groups. The rats were not fed for 24 hours before the commencement of the experiment. During the experiment, the rats were fed daily at 10 AM., for 22 days. A day before the end of the study (day 24), the animals were fasted.

A suspension of MPs was prepared by mixing 1g of the MPs in 1L of deionized water. Agglomeration of the MPs in suspension was assessed by transmission electron microscopy (JEOL JEM-1230 TEM). DLS-dynamic light scattering was utilized to determine the size range of particles. MPs feed-mixture was prepared and used throughout the growing period by mixing basal feed (55-70 g) with different concentrations of MPs (0, 5, 50, 100, 500, and 1000 μg/day).

2.3 Histological Analysis of Rat Tissues

After 24 days the rats were euthanized by carbon monoxide (CO) gas. The livers and the intestines were removed immediately after euthanasia. Tissue samples for light microscopy (LM) were fixed in 10% buffer formalin, then dehydrated in a series of ethanol and finally embedded in paraffin blocks. The tissues were then sectioned at 2-5 µm thickness and stained with Hematoxylin and Eosin stains (H&E). For TEM, small pieces of the tissue were fixed in Karnovsky (pH of 7.4) for 24 hours at 4 °C, then washed in sodium cacodylate buffer. Tissues were then post-fixed in 1% Osmium tetroxide washed and dehydrated in a series of ethanol solutions before being embedded in Agar 100 resin. Semi-thin sections (0.5 µm) were cut, stained with toluidine blue, and examined under a light microscope. Ultra-thin sections (60-90 nm) were cut and stained with uranyl acetate and lead citrate. Sections were examined under TEM (JEOL JEM-1230).

2.4 Gut microbiome analysis

Four rats were randomly selected from each treatment for analysis of the gut microbiome. The intestinal contents of the gut were removed using sterile techniques and genomic DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN). The DNA extracts were analyzed by paired-end Illumina MiSeq sequencing of the bacterial 16S rRNA genes V4 variable region using the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') with a barcode on the forward primer. The PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. DNA samples were then purified using calibrated AMPure XP beads. The purified PCR products were used to prepare a DNA library by following the Illumina DNA library. Sequencing was performed on a MiSeq following the manufacturer's guidelines. Sequence data were processed using the MR DNA analysis pipeline. In summary, sequences were joined, depleted of barcodes then sequences <150 bp and sequences with ambiguous base calls were removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from RDPII and NCBI (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu).

2.5 Statistical analysis

The effect of MPs on rat mass was analyzed by one-way ANOVA. The effects of treatment on villi conditions were analyzed by nested one-way ANOVA. For the bacterial community analysis, OTUs richness and Chao 1 index were calculated based on equal subsets of sequences for all samples (to the lowest number of sequences found in any sample), using an R-customized script. Non-metric multidimensional scaling (NMDS), based on Bray-Curtis dissimilarities of relative sequence abundances of OTUs, was conducted to visualize shifts in bacterial community composition with increasing concentrations of MPs. The extent of correlation between bacterial classes and MPs concentration was determined using the R software (v 3.4.1, R Core, 2017). A customized R script based on the correlate 0.84 package (R Studio v 3.5.1), was used to calculate the Pearson correlation coefficient (r). All the results were calculated as mean± standard deviation. The value of significance was considered when $p> 0.05$.

3. Results

3.1 Histological changes in rat tissues

Rat tissues exhibited clear modifications upon exposure to varying concentrations of MPs. The sinusoids of the liver enlarged and the gaps between the hepatic tissues expanded as the MPs concentration increased (Figure 1A & 1B). Intestinal mucosa had a normal appearance; however, some changes were observed within the villi of the intestinal mucosa, particularly at 500 and 1000 μg/L (Figure 2A & 2B).

An increase in peroxisomes was observed at 50 μg/L MPs (Figure 3A&3B). At 100 μg/L, autophagic vacuoles were also observed in the cells, especially near the endoplasmic reticulum. There was also some fragmentation and dilation of the endoplasmic reticulum. At 500 µg/L, the tissue showed irregular distribution of mitochondria and swelling cisternae of the endoplasmic reticulum. At 1000 µg/L, myeloid inclusions were abundant (Figure 3B). Some myeloid bodies could be seen inside hepatocytes. There was a loss of parallel arrangements in the rough endoplasmic reticulum. Several regions of the nuclear membrane and nuclei were damaged in the intestinal tissue as well. Cell death was observed at 500 and 1000 µg/L and the endoplasmic reticulum was fragmented (Figure 3B). Furthermore, the projections of the cellular surface to the neighbouring cells were discontinuous.

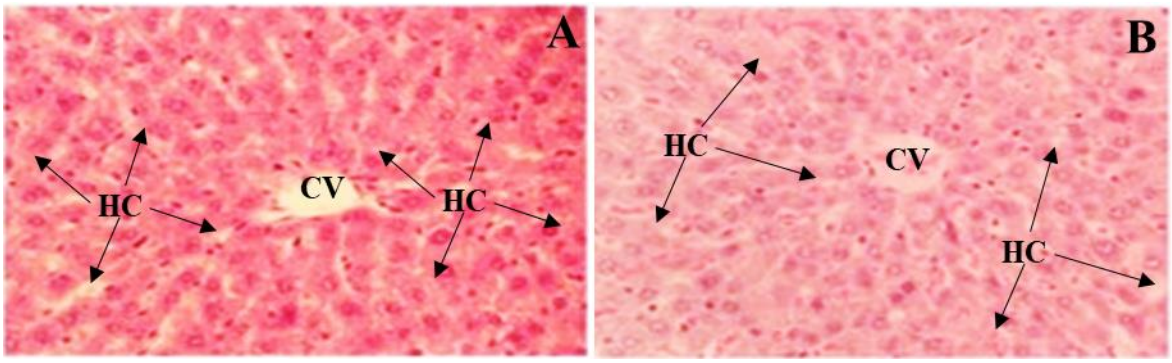

Figure 1. Light micrograph showing hepatic cells: Control (A) vs 100 µg/L of treated microplastic (B), hepatic cord (HC), central vein (CV). 400X, H&E.

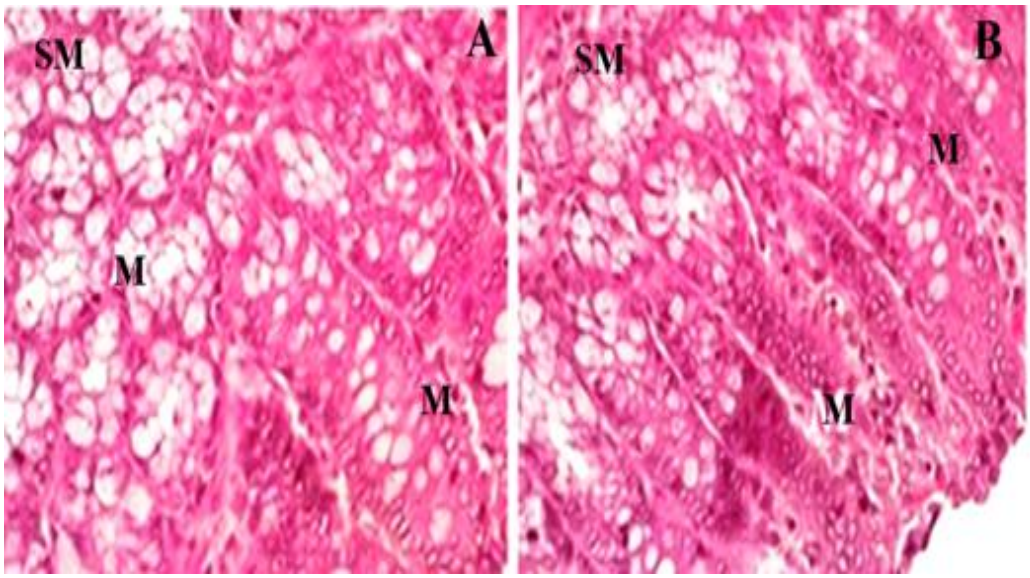

Figure 2. Light micrograph of intestinal mucosa: showing the control (A) vs 100 µg/L of treated microplastics (B) showing mucosa (M), submucosa (SM). 400X, H&E.

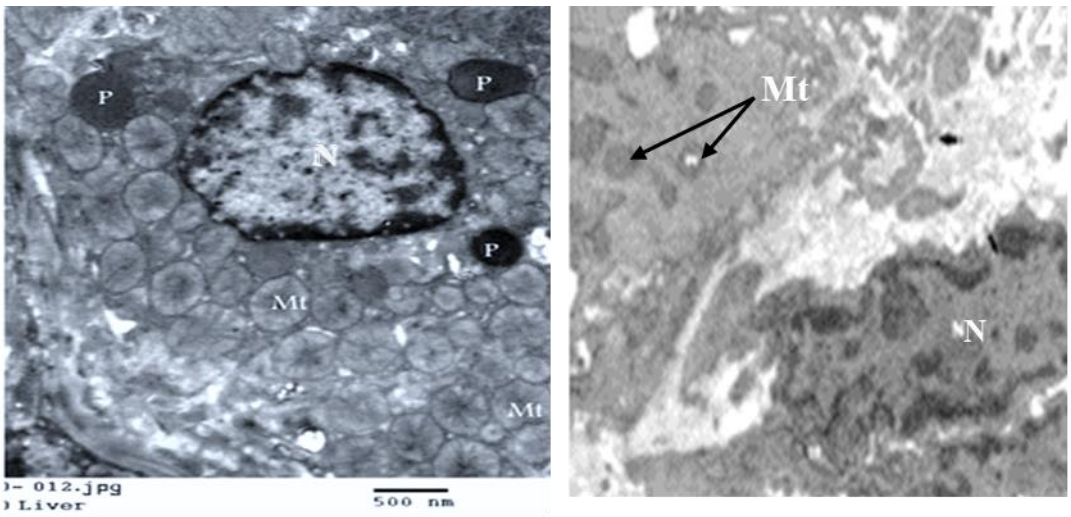

Figure 3. TEM micrograph of oxidative stress signs in the liver treated with µg/L (A) and intestine treated with 100 µg/L (B). Peroxisomes (P), mitochondria (Mt), Nucleus (N).

3.2 Shifts in Gut Microbiome

NMDS analysis of OTUs based on Bray-Curtis dissimilarities showed a clear heterogeneity in the bacterial community composition (Figure 4). The NMDS ordination based on the treatments with different concentrations of MPs did not show the formation of any distinct clusters and all samples were scattered (Figure 4). The diversity indices including OTU richness and Chao1 showed an increasing trend with increasing MPs concentration, with significantly higher values in the microbiomes exposed to MPs than in the control (except in the case of 50 μg/L, Figure 4B and C).

The gut microbiome was mainly composed of phylum Firmicutes, with an average relative abundance of 88±7% (Figure 4A). The Actinobacteria, Erysipelotrichia, and Betaproteobacteria were also detected (Figure 4A). At the class level, a significant linear increase in the relative abundance of Clostridia was observed with increasing concentrations of MPs (Figure 4B, R=0.036, P=0.003). Pearson correlations with the bacterial groups Bacteriodia, Bacilli, Actinobacteria, and Betaproteobacteria were not significant (Figure 5B, $p > 0.05$). Bacteriodia comprised between 24 to 28% of total sequences in the control, and this relative abundance remained more or less unchanged, except in the microbiome of rats exposed to 50 µg/L MPs, where the average relative abundance increased to 36.6±3% (Figure 5A).

Most of these sequences belonged to *Prevotella* spp., *Bacteroides sartorii* and a human fecal clone (Figure 6). None of hese sequences had any significant correlation with MPs concentration (not shown, $p > 0.05$). Clostridia exhibited its highest proportions in the microbiomes of rats treated with 500 and 1000 μg/L MPs and reached an average of 41±5% of total sequences (Figure 6A). The proportion of Clostridia in the remaining treatments, including the control was between 20% to 34% of total sequences. Clostridia were mainly affiliated with *Romboutsia johnsonii*, *Ruminococcus callidus*, and *Eubacterium coprostanoligenes* (Figure 6). Unlike Clostridia, the relative abundance of Bacilli in the microbiome of the control rats reached more than 42% in most replicates (3 out of 4). In all microbiomes exposed to MPs, at least three replicates had Bacilli at the relative abundance of less than 30% of total sequences, and only one replicate reached 40% of total sequences (Figure 6A). Bacilli included the most abundant OTU in the whole dataset (i.e. OTU1), which was related to *Lactobacillus faecis* (Figure 6). Pearson correlation showed that the relative abundance of this OTU significantly decreased with increasing concentration of MPs (not shown, r=-0.43, p=0.036). The second dominant Bacilli OTU was affiliated with *Lactobacillus johnsonii*, which did not show any significant change at the different concentrations of MPs. Actinobacteria constituted less than 20% of total sequences in all replicates of all treatments, with clear variation among replicates within each treatment (Figure 6A). The proportion of Actinobacteria in all replicates did not exceed 6% of total sequences at 1000 μg/L of MPs. Actinobacterial sequences were mainly related to the species *Bifidobacterium pseudolongum* (Figure 6B). Sequences belonging to Erysipelotrichia and Betaproteobacteria remained subdominant in the microbiome of all rats and their relative abundance was ≤7% of all sequences, regardless of the treatment (Figure 6A).

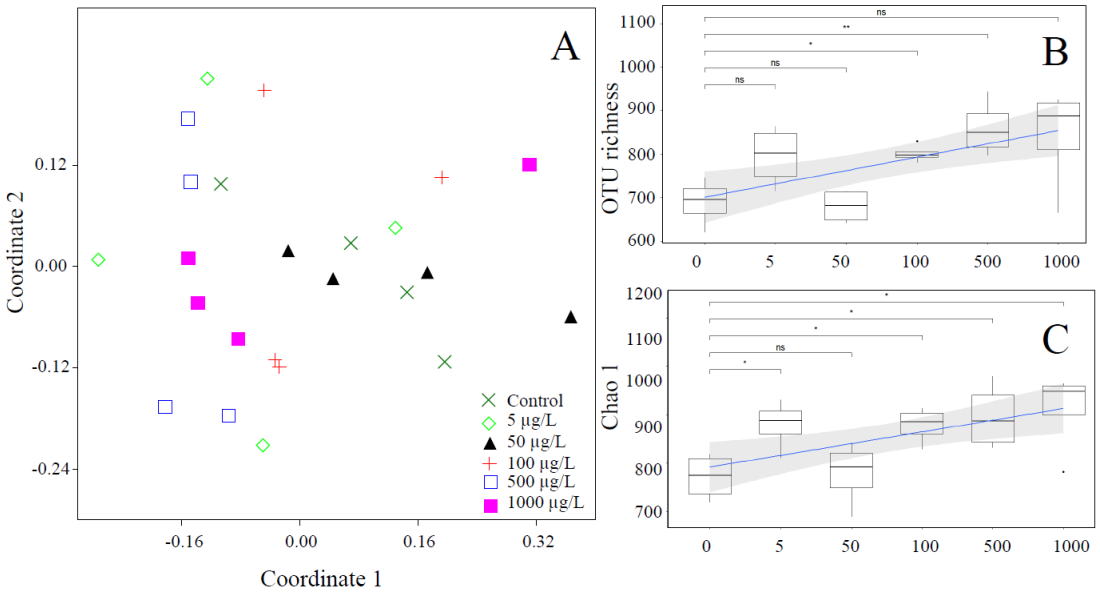

Figure 4. (A) Non-Metric Dimensional Scale (NMDS) showed the groups clustering in 3D for microbial communities in the sex groups for all animals. (B): OUT richness index (0 µg/L, 5 µg/L, 100 g/L, 500 µg/L and 1000 µg/L). (C): Chao1 index (0µg/L, 5µg/L, 50µg/L, 100 µg/L and 1000 µg/L). The boxplot illustrated interquartile range and inside the box, a line denotes the median, star indicate significant values (p≥0.05).

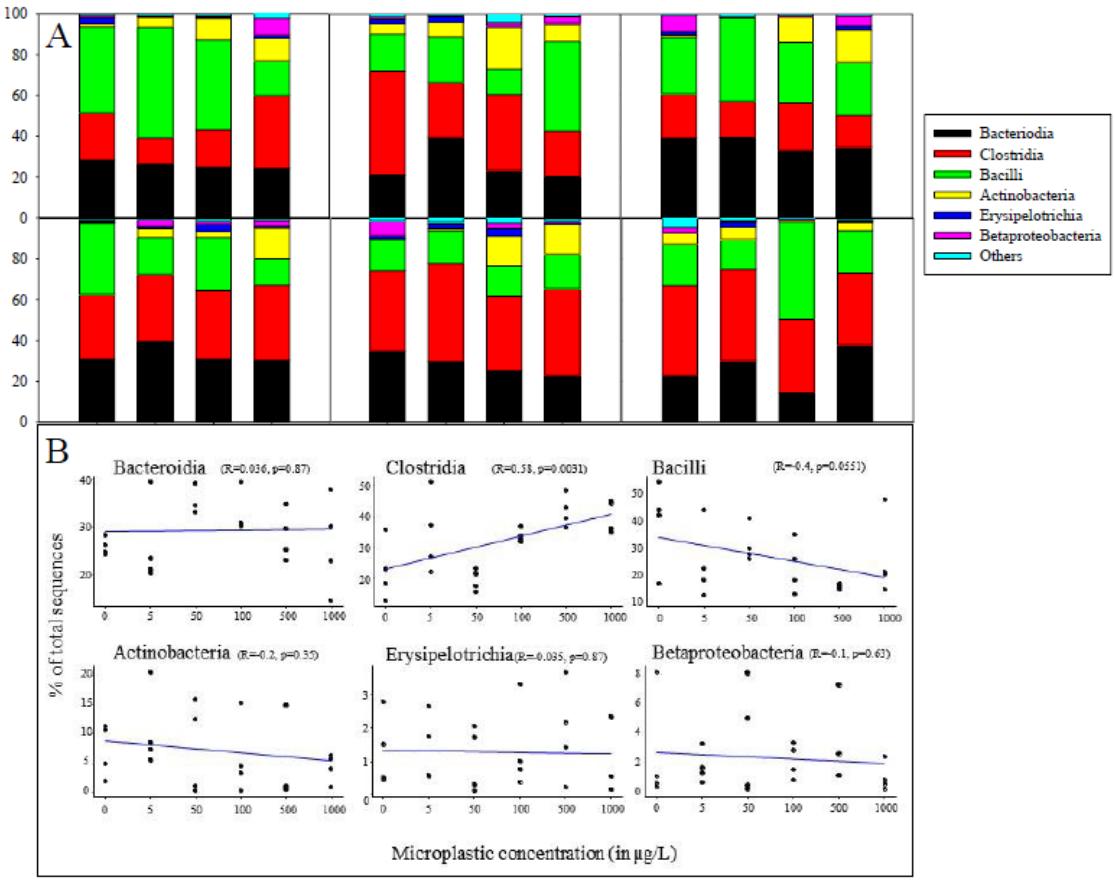

Figure 5. (A) Community barplot chart display the relative abundance (%) of main groups of bacteria Flora in rats intestine in highest dose of MP in treated groups (100 µg/L, 500 µg/L and 1000 µg/L). (B) Community barplaot Chart display the relative abundance (%) of main bacterial classes, (p) Pearson correlation with the bacterial groups, a significant linear correlation in MP treated groups (0, 5 µg/L, 50 µg/L, 100 µg/L, 500 µg/L and 1000 µg/L).

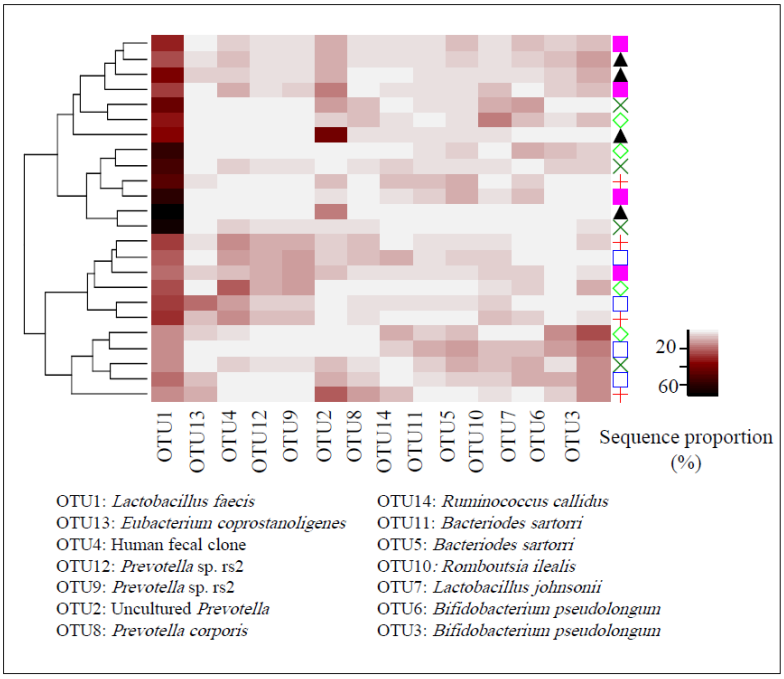

Figure 6. Heat map of OUTs number reveal the bacterial diversity and relative Abundance of bacterial species in all sequence in intestinal microbiome in all treated groups (0, 5 µg/L, 50 µg/L, 100 µg/L, 500 µg/L and 1000 µg/L).

Discussion

Our study demonstrated that the exposure to MPs induced changes in rats' tissue morphology as well as their gut microbiome. The toxicological impacts of MPs have been shown to depend on their size and dimension [5,25,26]. Smaller fragments of MPs tend to be more toxic than larger fragments due to their rapid translocation through the bloodstream into tissues [27,28]. MPs in the range of 5 to 280 µm were used to mimic the environment's prior distributions [29]. Although MPs had no significant effect on rat body weight, they were found to cause significant damage in the liver tissue, with an evident decrease in goblet cells in the crypts area.

The observed modifications in morphology and ultrastructure of rat tissues could be considered signs of oxidative stress. The increasing abundance of peroxisomes at 50 and 100 µg/L MPs indicated that oxidative stress increased with increasing concentrations of MPs. Peroxisomes have the ability to detoxify and metabolize amino acids [30], as well as to produce and scavenge reactive oxygen and nitrogen species [15,16,20,30,31]. The observation of lamellar inclusions in the epithelium of tissues of rats fed with 1000 µg/L MPs could be due to the high concentration and indigestibility of MPs, which resulted in their accumulation in the lysosome or late endosome as lamellar inclusions [20]. When the necrotic membrane ruptures, proteolytic enzymes are released, causing damage to cytoplasmic organelles [32,33]. Consequently, macroautophagy turnover can be slowed down [20,32], and myeloid inclusions can develop over time [20, 33]. High doses of MPs in rats exhibited detectable damage in lipid bilayers of plasma membranes [20]. This demonstrates that MPs can disrupt extracellular matrix components and proteoglycans [20]. MPs can also interfere with the cellular signaling process through interactions with cell surface receptors or extracellular ligands [21,31]. Previous studies have demonstrated that the fine MPs particles interfere with transport carrier trafficking [18,34] and inhibit signaling receptor expression [18,34].

The MPs had a significant impact on gut microbiome and could induce changes that may eventually influence animal health. It has been previously demonstrated that the gut microbiome is shaped by a range of factors [31], including intrinsic host characteristics and environmental factors, and is often linked to infections and chronic diseases [19]. Exposure to plastics (such as PE) altered the composition and diversity of gut microbiomes [19,20]. In our case, the bacterial diversity as indicated by Chao1 and OTU richness indices revealed an increasing trend with increasing concentration of MPs. This could indicate that MPs have changed the gut microenvironment in favor of new bacterial species. The increasing trend in the abundance of Clostridia and their higher abundance at 500 and 1000 µg/L corroborates this assumption. Clostridia are known to include strictly anaerobic species [36], able to form endospores, chemoorganotrophic, and can ferment a variety of nutrients [35,36]. Fermentation of organics by Clostridia spp. results in the production of acetic acid, butyric acid, and propionic acid, which play a vital role in intestinal homeostasis [36,37,38]. A vital fuel for colon cells (95% of butyrate), absorbed within colonic mucosa is produced by anaerobic butyrogenic bacteria like *Roseburia intestinalis* and *Faecalibacterium prausnitzii* [36]. Interestingly, it inhibits anti-inflammatory cytokine expression by facilitating hyperacetylation of chromatin [24]. The antioxidant properties of butyrate acid also protect against colitis and colorectal cancer.

The detection of *Lactobacillus faecis* as the most dominant OTU in the whole dataset highlighted its importance in the rat gut microbiome. The probiotic *Lactobacillus* species promoted systemic and mucosal immunity [24,36]. Therefore, the decreasing trend of this bacterium with increasing MPs concentration could mean that MPs influenced the rat immunity system. In recent years, it was has been demonstrated that the immune system responds to changes in the structure and metabolism of the gut microbiome [39,40]. It is believed that chronic inflammation is caused by lipopolysaccharides produced by the gut microbiome that are transported into the bloodstream [39,40]. Several diseases are associated with these compounds, including chronic liver disease, diabetes, ulcerative colitis, and Crohn's disease [7]. This suggests that MPs could influence the microbiome in a way that causes many disorders.

Actinobacteria seem to play a critical role in the gut microbiome since it constitutes one of the four major detected phyla. Bacteria belonging to this phylum are crucial in maintaining gut homeostasis [20]. Actinobacteria enhance tight junction expression, maintain intestinal barrier durability, regulate mucin biosynthesis and catabolism, provide energy to epithelial cells, and stimulate the immune system [21]. Despite the differences between treated groups, the significant decrease in the relative abundance of Actinobacteria at 1000 µg/L MPs concentration could indicate an increase in the gut permeability of rats.

Conclusions

MPs are becoming a global issue and can directly or indirectly affect organisms in the trophic chain. MPs can disrupt an organism's hormonal and physiological balance, depending on the type and concentration of MP as well as the duration of exposure. The presence of cytotoxicity and histopathology may develop over time following long-term exposure. A significant alteration can also occur in the gut microbiome; however, it is still unknown how these changes can affect the organism's physiology and health. There is a need to measure naturally aged PET in wild animals.

Conflict of interest

The authors declare no conflict of interest

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Ethics statements

This study has been approved by Sultan Qaboos University (SQU) Animal Ethics Committee (SQU-AEC-2019-20/10).

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