nature portfolio

Peer Review File

Polystyrene Nanoplastics Disrupt the Intestinal Microenvironment by Altering Bacteria-Host Interactions through Extracellular Vesicle-Delivered microRNAs

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Given the potential risk of nanopalastics (NP) on human health, this study is both intriguing and signficant to understand the mechanisms by which NP nanoparticles impact the gut barrier integrity and gut microbiota. Hsu and coauthors demonstrate here that NP nanoparticles interfer with tight junction proteins expression via their effect on the host intestinal and EV microRNAs.This NP-induced alteration in EV content of microRNAs in turn induces gut microbiota dysbiosis. Also, EV of some microbiota members exposed to NP suppress MUC-13 expression. The study is signficant to the field, however, the study needs a major revision before I can judge the research reported.

Major comments:
1-The manuscript requires an extensive revision of the English language.
2- Nothing is mentioned about the statistics used to reach the signficance of the results.
3- Methods details regarding sequencing libraries preparation and raw data analyses are completely missing, so I can not comment on the quality of the findings of these parts.
4- in many locations the authors refer to the figures without stating the findings, instead, they state the method approach the did. this affects the flow of the text.

Minor omments:
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2-lines 114-116: develop more on which genes are affected by NP.
3-lines 174-180: revise the English and rewrite this paragraph.
4-lines 186-190: Species number has no meaning unless they are generated from randomly selected equal number of sequences from every sample whichis unclear in the described method.
5-Why Simpson is used in alpha diversity over Shannon index and did you perform a PERMANOVA test for betadiversity.
6- Six mice is a low number for microbiota characterization
7- Are the increase or decrease in microbota abundance signficant? what statistical test you employed?
8- GIT transit time in mice is more than 2 hours, so why you collected mice feces after 2 hours of oral adminstration to check for NP uptake by bacteria?
9- identify AB-PAS at the first mention.

Reviewer #2

(Remarks to the Author)
The study is novel and reveal the toxic mechanism of NPs combined with NPs and EV miRNA. Some results were obtained, but it is not sufficient to be published in this journal. More evidence may be needed to be confirm this conclusion.
Line 64: This sentence is quite incorrect. There are many reports about the biological hazards caused by MP or NP. Please read the relevant articles and revise this sentence.
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Line 92-95: In the case of body weight gain, why the tissue weight and intestine length did not change? How to explain this result? Liver weight may be increased and should be added in the result. Why did the author test serum ALT, AST, CRE, BUN? These indexes indicate liver and kidney function.
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Figure 4CD: Why not use the same method to analyze MUC13 expression? And three parallels have different protein bands. The target protein bands do not correspond to the butler protein bans. Please confirm the reliability of the experimental results again. All the figure should be labeled with content of the experiment, for example, the immunohistochemistry of MUC13 (Figure 4B). At 24h, the expression of ZO-1 and Occludin as decrease, why only study the impact of 48 h later? And why choose this time point?
Line 165-167: How to determine that these miRNAs target MUC13 gene? How many times did miR-7a-5p rise? The heatmap did not do a good job of showing the result.
Line 183-188: The previous results were the experiments of mice exposed for 12 weeks. The fecal flora data of 4 weeks and 8 weeks were presented here, and there was no significant change. Why did it suddenly change after exposure for 12 weeks? Is there any regularity in the flora changes at 4, 8, and 12 weeks?
Figure 5: How many parallels there are in each treatment group? Please mention it in the figure caption. The data of β diversity can be analyzed with a cluster analysis, PCoA or PLSDA. And the data differences were not significant, in terms of beta diversity and α diversity (Figure 5BC).
Figure 6: There are no columns of Lachnospiraceae in LS174T cell-EV (+NP) at 0 day (Figure 6DE). Is that wrong?
Line 265: The morphology of the EVs in SEM do not have the characteristic appearance, such double-layer film. The pictures were very unclear.
Line 270-291: The evidence for the result is weak. The authors did not adequately demonstrate that gut microbiota is influenced by host cells. As the results showed, the Ruminococcaceae sp. were affected, but the Lachnospiraceae sp.-derived EV did not affect the MUC13 expression. How to explain this results?
The authors should examine the level of EVs in the intestinal contents to better conclude that NP is capable of altering EVs. The in vitro experiments revealed that NP changes EVs. The authors should also demonstrate in vivo that the EVs of these bacteria also have effects on the host's gut function or microenvironment.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Given the potential risk of nanopalastics (NP) on human health, this study is both intriguing and signficant to understand the mechanisms by which NP nanoparticles impact the gut barrier integrity and gut microbiota. Hsu and coauthors demonstrate here that NP nanoparticles interfer with tight junction proteins expression via their effect on the host intestinal and EV microRNAs.This NP-induced alteration in EV content of microRNAs in turn induces gut microbiota dysbiosis. Also, EV of some microbiota members exposed to NP suppress MUC-13 expression. The study is signficant to the field and the authors have addressed my concerns raised in the first revision, So I recommend accepting the manuscript for publication in its current format.

We would like to express our sincere gratitude to the Editor and Reviewers for their insightful comments and constructive feedback on our manuscript. We have carefully considered each suggestion and revised the manuscript accordingly, as detailed in our responses below. We appreciate the time and effort invested in reviewing our work and hope that the improvements we have made will meet the standards of *Nature Communications.*

Reviewer #1 (Remarks to the Author):

Given the potential risk of nanopalastics (NP) on human health, this study is both intriguing and signficant to understand the mechanisms by which NP nanoparticles impact the gut barrier integrity and gut microbiota. Hsu and coauthors demonstrate here that NP nanoparticles interfer with tight junction proteins expression via their effect on the host intestinal and EV microRNAs.This NP-induced alteration in EV content of microRNAs in turn induces gut microbiota dysbiosis. Also, EV of some microbiota members exposed to NP suppress MUC-13 expression. The study is signficant to the field, however, the study needs a major revision before I can judge the research reported.

Ans: We would like to thank Reviewer #1 for the thorough review and valuable suggestions on our manuscript. We have carefully considered all the comments and made the necessary revisions as outlined below.

Major comments:

1-The manuscript requires an extensive revision of the English language.

Ans: We appreciate this observation and have thoroughly revised the manuscript to improve grammar, syntax, and clarity. The revised version has been carefully edited to enhance readability and coherence.

2- Nothing is mentioned about the statistics used to reach the signficance of the results.

Ans: Thank you for pointing out the importance of providing detailed statistical analyses. We apologize for the omission and have now included detailed descriptions of the statistical methods used in our study. Specifically:

- Alpha diversity (Simpson index) was calculated using QIIME2, with group differences assessed via t-tests and Wilcoxon tests.

- Beta diversity was analyzed using UniFrac distance metrics and visualized with PCoA and PLS-DA plots in R. Statistical significance of beta diversity differences was assessed using t-tests and Wilcoxon tests.
- Taxa differences were analyzed using Welch's t-test in STAMP and permutation tests in R's metagenomeSeq package. P-values were corrected using the Benjamini and Hochberg False Discovery Rate method.

We have performed additional Beta Diversity Group Significance analyses based on similarity distance matrices, including ANOSIM and MRPP. The statistical analyses results indicate significant differences between two groups, as shown below:

- ANOSIM for two groups: p = 0.013
- MRPP for two groups: p = 0.008

These statistical methods have also been clearly detailed in the revised Methods section (lines 601-620).

3- Methods details regarding sequencing libraries preparation and raw data analyses are completely missing, so I can not comment on the quality of the findings of these parts.

Ans: We acknowledge this oversight and have now added comprehensive descriptions of microbiota sequencing and data analysis procedures in the Methods section (lines 601-620).

- Microbiota sequencing and data processing

  Sequencing data were demultiplexed based on barcode and primer sequences using QIIME2 (v2020.11; https://qiime2.org/). Primer sequences (forward: CCTACGGGNGGCWG; reverse: GACTACHVGGGTATCTAATCC) were removed using the QIIME2 cutadapt plugin, and the resulting sequences were denoised with QIIME2 DADA2. Reads were filtered (forward reads: 280 bp; reverse reads: 220 bp; MaxEE: 2), merged with a minimum overlap of 12 bp, and chimera sequences were removed. Amplicon sequence variants (ASVs) were generated and annotated using the classify-sklearn method trained on the SILVA (v132) or GreenGenes (gg_13_8) databases. Sequences were aligned using QIIME2 alignment MAFFT, and a phylogenetic tree was constructed with QIIME2 phylogeny fasttree. ASV tables were normalized to the smallest sample size before diversity analyses.

- Alpha diversity analysis

  Alpha diversity indices, including the Simpson index, were calculated using QIIME2 and analyzed for group differences with parametric (t-test) or non-parametric (Wilcoxon) tests. Diversity metrics were visualized with rarefaction curves generated in R (v3.3.1).

- Beta diversity analysis

  Beta diversity was evaluated using principal coordinates analysis (PCoA) and partial least squares discriminant analysis (PLS-DA) plotted in R with the ade4, mixOmics, and ggplot2 packages. Statistical significance of beta diversity differences between groups was determined using parametric (t-test) and non-parametric (Wilcoxon) tests in the agricolae R package.

- Differential taxa analysis

  Species-level differences were identified using Welch's t-test in STAMP (v2.1.3) and visualized with bar charts. MetagenomeSeq in R was employed for permutation tests, with p-values adjusted using the Benjamini and Hochberg False Discovery Rate method to calculate q-values. Statistically significant taxa differences ($p/q < 0.05$) were presented as boxplots. Anosim and MRPP analyses were conducted using the vegan R package.

We hope these additions and revisions address the reviewer's concerns effectively.

4- in many locations the authors refer to the figures without stating the findings, instead, they state the method approach the did. this affects the flow of the text.

Ans: We appreciate this valuable feedback and have revised the manuscript to clearly state the findings associated with each figure, rather than focusing solely on the methods. These changes aim to improve the flow of the text and enhance the clarity of our scientific narrative.

Minor omments:

1- the term"flora" does not sound scientific term anymore. Please replace it with microbiota.

Ans: Thank you for your correction. We have replaced all instances of "flora" with "microbiota" throughout the manuscript.

2-lines 114-116: develop more on which genes are affected by NP.

Ans: Thank you for your valuable suggestion. We have described as **“**Transcriptome analysis was performed in this study to evaluate the impact of NP on host intestinal function. The volcano plot of differentially expressed genes illustrates individual genes, with significant up-regulated and down-regulated genes (Log_2(FC) > 2 and *p*-value < 0.05) highlighted in green and red, respectively **(Supplementary Fig. 1A)**. A Heatmap displayed the differentially expressed protein-coding genes that were up-regulated by NP treatment, with criteria set at a twofold expression change and *p*-value < 0.05, along with Gene ontology (GO) functional enrichment **(Supplementary Fig. 1B)**. Similarly, a heatmap showed the protein-coding genes down-regulated by NP treatment under the same criteria, and GO analysis revealed that NP exposure significantly affected intestinal gene expression and metabolic functions in mice **(Supplementary Fig. 1C)**. Gene expression is often regulated by microRNAs (miRNAs), short non-coding RNAs typically 18-23 nucleotides in length. To explore the impact of NP on the intestinal host, we examined miRNAs and their regulatory roles. Although previous studies have quantified RNA in human stool and identified miRNAs as potential indicators of the intestinal environment [22,23], the presence and function of fecal miRNAs in the normal gut remain largely unexplored. In this study, we identified intestinal miRNAs and investigated their role in shaping gut microbiota composition. Principal component analysis (PCA) of miRNA diversity in mouse feces revealed that NP treatment significantly altered miRNA profiles and reduced miRNA diversity **(Fig. 3A)**. The specific miRNAs that were significantly upregulated by NP exposure are shown in **Fig. 3B**. Further analysis demonstrated that these miRNAs regulate key physiological functions, including those related to intestinal cell junctions (GO: 0034329) **(Fig. 3C)”.** (lines 124-147)

3-lines 174-180: revise the English and rewrite this paragraph.

Ans: Thank you for your suggestion. We have rewritten the paragraph and described as “Several hundred miRNAs have been identified in exosomes and extracellular vesicles (EVs) derived from feces and edible plants, with their profiles analyzed for their potential to target and regulate genes in various hosts [30-32]. Beyond miRNAs, EVs derived from intestinal cells, the composition of mucus within the intestinal environment, and interactions among microbial communities are critical factors influencing intestinal microbiota composition and overall host health [30-34]. While

numerous studies have shown that nanoplastics (NP) and microplastics (MP) contribute to gut microbiota imbalance, the mechanisms driving these disruptions remain poorly understood [1-8].” (lines 206-214).

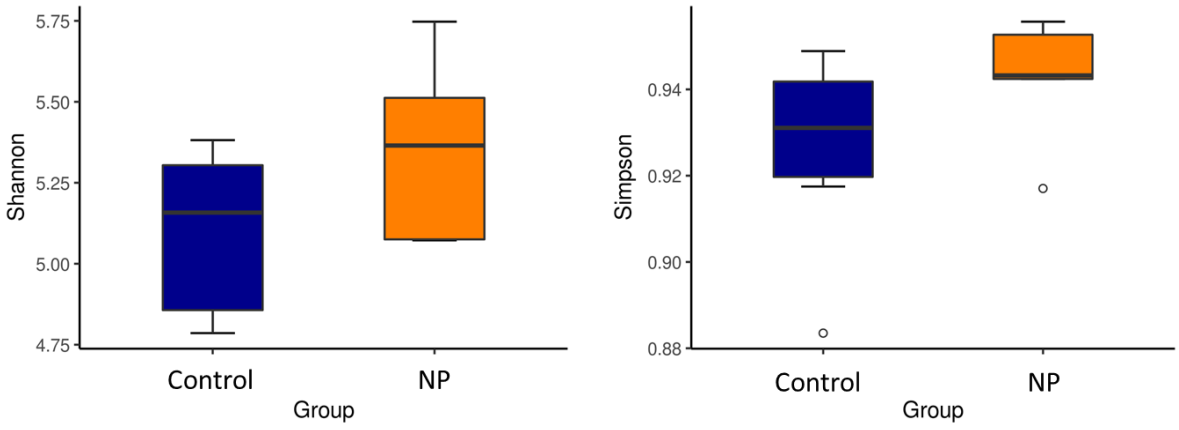
4-lines 186-190: Species number has no meaning unless they are generated from randomly selected equal number of sequences from every sample whichis unclear in the described method.

Ans: We appreciate the reviewer’s comment regarding the interpretation of species numbers. To clarify, we have addressed this concern in the manuscript. Specifically, we described the findings as follows: “Compared to the control group, the NP treatment group displayed an increase of 112 unique bacterial species after 12 weeks of treatment, as shown in the Venn diagram (Fig. 5A). At earlier time points, 171 and 161 unique bacterial species were identified after 4 weeks (Supplementary Fig. 4) and 8 weeks (Supplementary Fig. 5) of NP treatment, respectively. While the number of unique bacterial species decreased with prolonged NP exposure, the top 10 bacterial families shared between the NP treatment and control groups-including *Ruminococcaceae*, *Lactobacillaceae*, and *Lachnospiraceae*-did not show significant changes in abundance. Interestingly, *Akkermansia*, a next-generation probiotic bacterium, exhibited a higher abundance in the NP treatment group compared to the control group, particularly from 8 weeks to 12 weeks of treatment. While the underlying mechanisms remain unclear, these observations suggest that NP treatment may exert a targeted and systematic effect on specific bacterial species (Supplementary Fig. 5)” (lines 217-229). We hope this explanation sufficiently addresses the reviewer’s concerns regarding the description and relevance of species numbers in our analysis.

5-Why Simpson is used in alpha diversity over Shannon index and did you perform a PERMANOVA test for betadiversity.

Ans: Thank you for your valuable suggestion. In this study, we also conducted Shannon index analysis in microbiota analysis, and the results were consistent with the Simpson index, showing no significant differences between the two groups. For simplicity and clarity, we opted to present the Simpson index as a representative alpha diversity metric in the manuscript. Regarding PERMANOVA, we acknowledge its

value; yes, we also did permutational MANOVA (Adonis) analysis. The statistical analyses results indicate significant differences between two groups: p = 0.05.



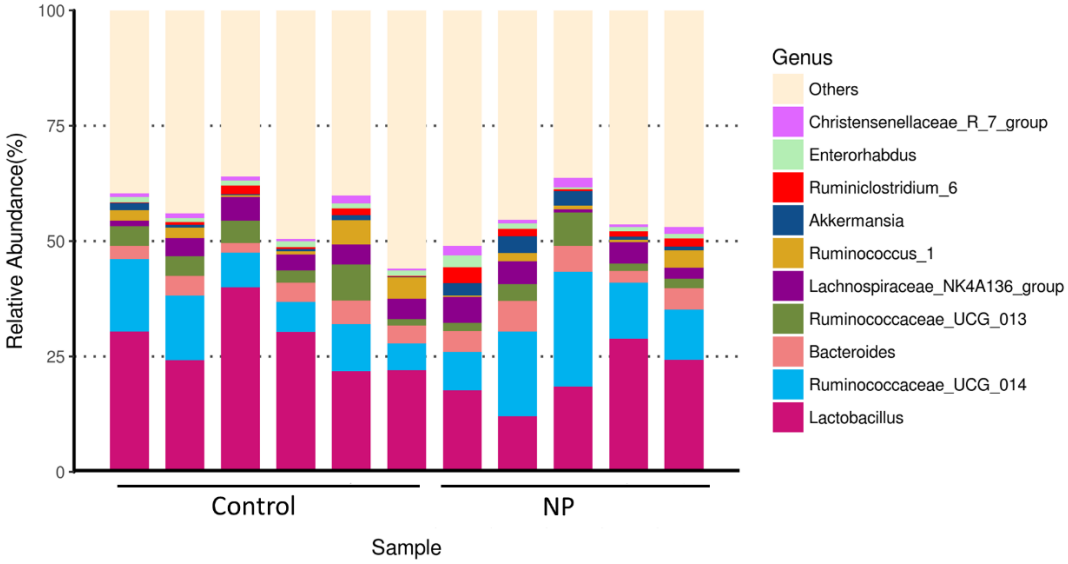
6- Six mice is a low number for microbiota characterization

Ans: We appreciate the reviewer's concern regarding the adequacy of n=6 for microbiota analysis. In designing the experiment, we ensured that the sample size met the statistical reliability criterion of having more than five samples per group. Despite this limitation, our results demonstrate that within-group variability is small, and between-group differences are greater than within-group differences. This is evidenced by the similarity in microbial composition among the six mice within each group, as shown in the genus-level relative abundance plot. The sequencing and analysis results demonstrated that the microbial community structures within each group were highly similar, indicating a stable experimental design and minimizing the risk of statistical inaccuracies due to sample size limitations.

Furthermore, we utilized Anosim and MRPP statistical analyses to assess whether between-group differences exceeded within-group variation. Both analyses confirmed that the differences between groups were statistically significant: Anosim (p-value = 0.013) and MRPP (significance = 0.008). These results support the robustness of our findings and validate the representativeness of the sample size used in this study. The Anosim analysis revealed a significant difference in microbial community structure between the control and NP treatment groups, with an R-value of 0.1680. An R-value greater than 0 indicates that between-group differences are greater than within-group differences, supporting the representativeness of n=6 in this study.

Additionally, these findings are supported by complementary results from other experiments, which collectively strengthen the validity of our conclusions. We hope

this explanation and the provided evidence address the reviewer's concern.



7- Are the increase or decrease in microbota abundance signficant? what statistical test you employed?

Ans: We appreciate the reviewer's inquiry. Statistical significance for changes in microbiota abundance was assessed using Welch's t-test in STAMP (v2.1.3). Additionally, permutation tests were conducted using the metagenomeSeq package in R, with p-values adjusted via the Benjamini and Hochberg False Discovery Rate method to calculate q-values. Only bacterial taxa with $p/q < 0.05$ were considered significantly altered. These methods have been detailed in the revised Methods section (lines 600-620).

8- GIT transit time in mice is more than 2 hours, so why you collected mice feces after 2 hours of oral adminstration to check for NP uptake by bacteria?

Ans: We appreciate the reviewer's comment and provide the following clarifications regarding this issue:

(1) Gastrointestinal transit time variability

Research indicates that the retention time of different substances in the gastrointestinal tract can vary significantly depending on their form and the fasting state of the animal. While solid food typically remains in the gastrointestinal tract for more than 2 hours, liquids are more influenced by intestinal peristalsis, potentially reaching the intestines within this timeframe (Laboratory Animal and Comparative Medicine, 2012, 32, 241-242). Based on

this, we hypothesize that liquid NP administered to fasting mice may reach the intestines within 2 hours.

(2) Feces collection methodology

In this study, feces were collected directly from the intestines 2 hours after gavage, following the sacrifice of the animals, rather than relying on naturally excreted feces. This approach ensures that the analysis reflects microbial interactions with NP in the intestinal environment.

(3) Literature support

The experimental design was informed by prior studies, including work published in Cell Host & Microbe (2018, 24, 637-652; Figure 1F), which provided valuable insights into similar methodologies.

We hope these explanations address the reviewer's concerns regarding the timing of feces collection and its relevance to the study.

9- identify AB-PAS at the first mention.

Ans: Thank you for pointing this out. We have clarified the identification of AB-PAS at its first mention in the manuscript. It is described as follows: "The Alcian blue-Periodic acid Schiff (AB-PAS) stain is widely used in histology to detect carbohydrates in tissues. Periodic acid oxidizes hydroxyl groups on adjacent carbon atoms in carbohydrate molecules, forming aldehyde groups that react with Schiff's reagent to produce a magenta or purple-red coloration." (lines 175-179).

Reviewer #2 (Remarks to the Author):

The study is novel and reveal the toxic mechanism of NPs combined with NPs and EV miRNA. Some results were obtained, but it is not sufficient to be published in this journal. More evidence may be needed to be confirm this conclusion.

Ans: We would like to thank Reviewer #2 for recognizing the novelty of our study and its contributions to understanding the toxic mechanisms of nanoplastics (NPs) and extracellular vesicle (EV) miRNA. We appreciate your feedback and have carefully considered your suggestion for additional evidence to strengthen our conclusions.

To address your concern, we have included new data to confirm our conclusions. Specifically:

1. Additional experiments: We have added Western blot data of MUC13, and we have conducted additional microbiota and miRNA statistical assays and validated key findings with qPCR to confirm the observed changes in bacterial composition and EV miRNA alterations.
2. Detailed statistical analysis: We have expanded the statistical methods employed to evaluate the significance of our results. For example, we used Welch's t-test and metagenomeSeq for differential abundance analysis, with p-values adjusted using the Benjamini and Hochberg method. These details have been incorporated into the revised Methods section.

Furthermore, we have enhanced the discussion to:

- Explicitly link our findings to existing literature, emphasizing the biological relevance of the observed changes in specific bacterial taxa and EV miRNA content.
- Address potential mechanisms underlying the observed effects of NPs and EV miRNA on gut microbiota and host physiology.
- Acknowledge the limitations of the current study and propose future research directions to validate and extend our findings.

We hope these additions and revisions provide a more robust foundation for our conclusions and address the reviewer's concerns effectively.

1. Line 64: This sentence is quite incorrect. There are many reports about the biological hazards caused by MP or NP. Please read the relevant articles and revise this sentence.

Ans: Thank you for your observation. We have revised the sentence to reflect the

existing body of research on the biological hazards caused by MP and NP. The updated text now reads: “NP, due to their nanoscale size, can penetrate tissues and organs, posing potential biological hazards. Yet, the specific pathways through which NP disrupt gut microbiota balance and impact intestinal health are not well characterized. To address this gap, this study investigates the biological accumulation of NP and their effects on the intestinal microenvironment, with a focus on bacteria-host interactions” (lines 61-66)

We hope this revision addresses the reviewer’s concern.

2. Line 89-90: So how is the concentration determined? What is the characterization of NP? And Why is NP being fed in this way, at a frequency of 4 times a week?

Ans: Thank you for the insightful question. Given that NP can persist in the colon for up to 48 hours, oral administration of polystyrene NP was conducted four times a week to mimic continuous exposure to NP while allowing for a recovery period between doses. This design ensures that the accumulation and effects of NP are evaluated in a manner representative of sustained, yet manageable, exposure over time.

We have addressed the rationale for the NP feeding frequency as follows: “Mice administered fluorescently (FluoSpheres carboxylate-modified microspheres) labeled NP (100 nm) displayed substantial NP accumulation in the cecum, liver, small intestine, and colon at 3, 6, 12, 18, 24, 36, and 48 hours post-administration **(Fig. 1A)**. These findings align with previous reports of NP persistence in the gut [17]. Given that NP can persist in the colon for up to 48 hours, this study further examined their effects on the intestinal environment through oral administration of polystyrene (100 nm; 2×10^{11} particles/mouse, concentration determined by Nanosight) four times a week (on days 1, 3, 5, and 7 of each week).” (lines 86-93)

We hope this revision clarifies the rationale for the NP concentration, characterization, and administration frequency.

3. Line 92-95: In the case of body weight gain, why the tissue weight and intestine length did not change? How to explain this result? Liver weight may be increased and should be added in the result. Why did the author test serum ALT, AST, CRE, BUN? These indexes indicate liver and kidney function.

Ans: Thank you for raising these important points. We have addressed these questions

in the revised manuscript as follows: “Recent studies have indicated that polyvinyl chloride-MP may induce hepatic injury [1], while PS-MP exposure has been linked to disruptions in blood triglyceride metabolism [17]. Additionally, PS-MP has been associated with obesity and increased adiposity in mice [18]. In this study, prolonged NP exposure through oral administration (four times per week for 12 weeks) resulted in an increased body weight in NP-treated mice compared to controls (5.17 g vs. 4.03 g; $p > 0.01$), although no significant changes in white adipose tissue weight or liver weight were observed **(Fig. 1B)**. The length of the intestine can be used as an indicator to assess the severity of intestinal inflammation [19]. Previous studies have confirmed that both MP and NP pose a threat to the intestinal environment and microbiota [1-8]. The absence of intestinal shortening in NP-exposed mice **(Fig. 1C)** suggests that intestinal bacteria, rather than inflammation, may be the primary target of NP-induced effects. Despite the established toxicity of MP on liver and kidney functions [1,17], NP exposure for 12 weeks did not significantly alter serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CRE), or blood urea nitrogen (BUN) levels **(Fig. 1D)**. This indicates that the intestinal microbiota and barrier may be more directly affected by NP.” (lines 94-110)

We hope this response addresses the reviewer’s concerns regarding body weight, tissue weight, intestinal length, and serum biomarkers.

Figure 1D: The legend is unclear, which should be adjust the size and mark.

Ans: We appreciate the reviewer’s observation. We have revised the legend for Figure 1D to improve its clarity by adjusting the size and formatting. We have also added appropriate markings to ensure the information is more easily interpretable.

Figure 3B: The heatmap of miRNA was repeated and the results was different? Just a heatmap could show the miRNA.

Ans: Thank you for highlighting this point. To investigate the effects of NP on intestinal microRNAs and their role in intestinal physiology, we evaluated the microRNAs significantly upregulated by NP treatment. In the original **Fig. 3B**, we included heatmaps showing both the results from six replicates and the mean values. In the revised version, we have simplified the presentation by retaining only the heatmap of the mean values to enhance clarity and focus. Additionally, we performed GO analysis to assess the pathways affected by these altered microRNAs, with the

results presented in **Fig. 3C**. These analyses collectively provide a comprehensive understanding of the changes in microRNAs and their physiological implications, demonstrating that the data cannot be fully explained by a single figure alone **(lines 135-162)**.

We hope this clarification addresses the reviewer's concerns.

Line 144-148: The miRNAs are highly conserved. Are changed miRNAs of mice consistent with those of human cells after exposure to NP? There is no identical change in miRNA.

Ans: We greatly appreciate the reviewer's insightful suggestion. This is indeed a question we have been investigating since the inception of our research. In our study, the assessment of microRNAs in mice was conducted using the entire intestine, which includes a variety of cell types. In contrast, for human cells, we specifically analyzed the LS174T cell line, which models goblet-like cells. We believe that directly comparing the results from mixed-cell mouse intestine samples to those from a single human cell line may not be appropriate due to the inherent differences in cellular composition and biological context.

Nonetheless, one of the key findings of our study is that NP reduces ZO-1 expression through the regulation of microRNAs. This observation is consistent across both models and represents a clear and significant result, providing valuable insights into the mechanisms by which NP disrupts intestinal barrier integrity (lines 135-169).

We hope this explanation addresses the reviewer's concerns.

Figure 4CD: Why not use the same method to analyze MUC13 expression? And three parallels have different protein bands. The target protein bands do not correspond to the butler protein bans. Please confirm the reliability of the experimental results again. All the figure should be labeled with content of the experiment, for example, the immunohistochemistry of MUC13 (Figure 4B). At 24h, the expression of ZO-1 and Occludin as decrease, why only study the impact of 48 h later? And why choose this time point?

Ans: Thank you for the reviewer's thoughtful comments. We have carefully addressed the points raised as follows:

(1) Adding new data for confirmation

To ensure the reliability of the results, we have supplemented the data using multiple methods. For **Fig. 4C**, we confirmed the immunocytochemistry (ICC) staining results with Western blot and qPCR analyses. Similarly, for **Fig. 4D**, we have re-verified the Western blot results. Additionally, for **Fig. 4F**, we corroborated the ICC staining results (**Fig. 4G**) with both qPCR (**Fig. 4F**) and Western blot analyses (**Fig. 4H**). These additional data strengthen the reliability and reproducibility of the findings.

(2) Consistency across figures

In **Fig. 3**, we also included Western blot data (**Fig. 3F**) to confirm the results obtained from ICC staining (**Fig. 3E**), further supporting the observations.

(3) Selection of time points

The decrease in ZO-1 and Occludin expression was observed as early as 24 hours after NP treatment. However, the reduction in MUC-13 expression occurred only after 48 hours of NP treatment. Therefore, we selected the 48-hour treatment period for the MUC-13 experiments to accurately capture this time-dependent effect. This rationale has been clearly explained in the figure legends.

(4) Protein band reliability

Regarding the differences in protein bands and their correspondence to the loading control (butler protein) bands, we have re-evaluated and confirmed the reliability of the Western blot results to address any discrepancies (**Fig. 3** and **Fig. 4**).

(5) Labeling figures

We have ensured that all figures are appropriately labeled with the content of the experiment. For example, **Fig. 4B** has been updated to include the label " MUC13" for clarity and consistency.

We hope these additional clarifications and revisions address the reviewer's concerns comprehensively.

Line 165-167: How to determine that these miRNAs target MUC13 gene? How many times did miR-7a-5p rise? The heatmap did not do a good job of showing the result.

Ans: Thank you for pointing out this issue. We have supplemented additional data and detailed explanations in the revised manuscript (lines 194-214) to address this

concern.

(1) Determining miRNA targets

The identification of miRNAs targeting the MUC13 gene was conducted using predictive bioinformatics tools, including hybrid prediction techniques. These analyses identified miR-7a-5p as a candidate miRNA with specific binding sites on the MUC13 gene.

(2) Fold change of miR-7a-5p

To clarify, the increase in miR-7a-5p expression after NP treatment was quantified and is now explicitly described in the revised text.

(3) Improved data presentation

The heatmap has been revised to better visualize the changes in miRNA expression. Additionally, we have included complementary data in **Fig. 4E** and Supplementary Figures to ensure clarity and provide a more comprehensive view of the results.

We hope these revisions and supplementary data sufficiently address the reviewer's concerns.

Line 183-188: The previous results were the experiments of mice exposed for 12 weeks. The fecal flora data of 4 weeks and 8 weeks were presented here, and there was no significant change. Why did it suddenly change after exposure for 12 weeks? Is there any regularity in the flora changes at 4, 8, and 12 weeks?

Ans: Thank you for raising this important question. We have carefully addressed this concern in the revised manuscript (lines 217-252).

(1) Temporal dynamics of microbiota changes

We observed that the microbial composition showed minimal changes after 4 and 8 weeks of NP exposure, as presented in the fecal microbiota data (**Supplementary Fig. 4** and **Supplementary Fig. 5**). However, significant changes in microbiota composition was noted after 12 weeks of exposure. These delayed effects suggest a cumulative or threshold-based impact of NP on the gut microbiota, which may not manifest during shorter exposure periods.

(2) Potential mechanisms for delayed changes

The significant changes observed after 12 weeks could be attributed to the progressive accumulation of NP in the gut and the resulting long-term

interactions with intestinal cells and microbiota. The time-dependent disruption of host-microbiota interactions, particularly through alterations in EV-mediated communication, may play a crucial role in these delayed changes.

(3) Regularity in microbiota changes

We have expanded the discussion to describe the regularity in microbial changes over time. Specifically, while the number of unique bacterial species decreased with prolonged exposure, certain bacterial families, such as *Ruminococcaceae*, progressively increased in abundance, particularly at 12 weeks. These findings indicate a gradual and systematic shift in the gut microbiota that becomes evident only after extended NP exposure.

We have detailed these findings and their potential implications in the revised manuscript and included relevant references to support our interpretation. We hope this explanation clarifies the observed patterns in microbiota changes over the 4-, 8-, and 12-week exposure periods.

Figure 5: How many parallels there are in each treatment group? Please mention it in the figure caption. The data of β diversity can be analyzed with a cluster analysis, PCoA or PLSDA. And the data differences were not significant, in terms of beta diversity and α diversity (Figure 5BC).

Ans: We appreciate the reviewer's detailed observations. Each treatment group includes six biological replicates. This information has been added to the figure caption of **Figure 5** to enhance clarity.

In this study, we used Simpson index for α diversity and used PCoA and PLSDA for β diversity analysis to evaluate microbial community structures because they allow clear visualization of group clustering and is commonly used in microbiome studies.

Regarding the reviewer's observation about the significance of differences in β and α diversity (**Figure 5BC**), we acknowledge that while the differences were not statistically significant, a high level of dispersion was observed between groups in β diversity. This dispersion reflects variations in microbial composition, suggesting that NP may exert indirect effects on the microbiota, potentially mediated through host modifications. Rather than inducing widespread alterations, NP appears to selectively influence certain bacterial groups.

We believe these observed trends offer valuable insights into subtle shifts in microbial community composition under NP treatment. These findings and their biological relevance have been discussed in greater detail in the revised manuscript. Additionally, the figure caption for **Figure 5** has been updated to provide a comprehensive explanation of the microbial composition results, including the observed high dispersion.

Figure 6: There are no columns of Lachnospiraceae in LS174T cell-EV (+NP) at 0 day (Figure 6DE). Is that wrong?

Ans: We appreciate the reviewer for pointing out this issue. The omission of the 0-hour value for *Lachnospiraceae* in the LS174T cell-EV (+NP) group during figure preparation was an oversight. We have now added the correct value, updated the figure (**Fig. 6**), and ensured its accuracy. The revised figure has been included in the manuscript.

Line 265: The morphology of the EVs in SEM do not have the characteristic appearance, such double-layer film. The pictures were very unclear.

Ans: We greatly appreciate the reviewer's insightful comments regarding the morphology of EV in the TEM images. It is indeed well-documented in the literature that the quality and appearance of images can vary depending on the specific TEM equipment used. The equipment currently available to us is limited in its ability to capture certain morphological features, such as the double-layer film characteristic of EV, resulting in the images presented. To address this concern, we have provided several references that show similar variations in EV imaging:

(1) Zhang Y, Sun C, Wang B, Gu A, Zhou Z, Gu C. Brain-Derived Exosomal miR-9-5p Induces Ferroptosis in Traumatic Brain Injury-Induced Acute Lung Injury by Targeting Scd1. *CNS Neurosci. Ther.* **30**, e70189 (2024). doi: 10.1111/cns.70189.

(2) Spinelli C, Montermini L, Meehan B, Brisson AR, Tan S, Choi D, Nakano I, Rak

J. Molecular subtypes and differentiation programmes of glioma stem cells as determinants of extracellular vesicle profiles and endothelial cell-stimulating activities. *J. Extracell. Vesicles* **7**, 1490144 (2018). doi: 10.1080/20013078.2018.1490144.

(3) Pultar M, Oesterreicher J, Hartmann J, Weigl M, Diendorfer A, Schimek K, Schädl B, Heuser T, Brandstetter M, Grillari J, Sykacek P, Hackl M, Holnthoner W. Analysis of extracellular vesicle microRNA profiles reveals distinct blood and lymphatic endothelial cell origins. *J. Extracell. Biol.* **3**, e134 (2024). doi: 10.1002/jex2.134.

(4) Liao Q, Su L, Pang L, Li J, Li H, Li J, Liu Y, Zhang J. Natural exosome-like nanoparticles derived from ancient medicinal insect Periplaneta americana L. as a novel diabetic wound healing accelerator. *J. Nanobiotechnology* **21**, 169 (2023). doi: 10.1186/s12951-023-01923-1.

(5) Setua S, Thangaraju K, Dzieciatkowska M, Wilkerson RB, Nemkov T, Lamb DR, Tagaya Y, Boyer T, Rowden T, Doctor A, D'Alessandro A, Buehler PW. Coagulation potential and the integrated omics of extracellular vesicles from COVID-19 positive patient plasma. *Sci. Rep.* **12**, 22191 (2022). doi: 10.1038/s41598-022-26473-8.

Line 270-291: The evidence for the result is weak. The authors did not adequately demonstrate that gut microbiota is influenced by host cells. As the results showed, the Ruminococcaceae sp. were affected, but the Lachnospiraceae sp.-derived EV did not affect the MUC13 expression. How to explain this results?

Ans: We appreciate the reviewer’s comment and have provided a detailed explanation to address this concern (lines 292-339).

In summary:

(1) NP-induced microbiota imbalance through host regulation

The imbalance in intestinal microbiota caused by NP is hypothesized to result from NP-induced dysregulation in the host intestine rather than direct bacteria-bacteria interactions. Prior studies have shown that specific miRNAs in extracellular vesicles (EV) regulate bacterial growth, with miR-1226-5p enhancing the growth of *Escherichia coli* and miR-515-5p promoting *Fusobacterium nucleatum*. This differential targeting by host-derived miRNAs supports the hypothesis that NP disrupts gut microbiota composition through host-microbiota interactions.

(2) Role of goblet-like cells and EV

Our study confirmed that NP treatment significantly increased EV production in goblet-like LS174T cells (**Supplementary Fig. 6**). EV derived from NP-treated goblet-like cells enhanced the growth and survival of *Ruminococcaceae* sp. (**Fig. 6E**), while EV from enterocyte-like Caco-2 cells, irrespective of NP treatment, had no similar effect (**Supplementary Fig. 7**). This indicates that goblet-like cells are more critical than enterocyte-like cells in regulating the gut microbiome under NP exposure.

(3) Contrasting effects of *Lachnospiraceae* and *Ruminococcaceae* EV

While *Ruminococcaceae* sp.-derived EV did not significantly alter MUC-13 expression (**Supplementary Fig. 8**), NP-treated *Lachnospiraceae* sp.-derived EV significantly inhibited MUC-13 expression in goblet-like LS174T cells (**Fig. 6F**). This suggests that NP influences *Lachnospiraceae* sp. differently, primarily through host microRNAs, as shown in **Fig. 4** and **Fig. 5G**.

(4) Biological relevance of *Ruminococcaceae* sp.

The growth of *Ruminococcaceae* sp. is particularly concerning, as its increased

abundance has been associated with Autism Spectrum Disorder (ASD), nerve damage, aging, and liver fibrosis. We hypothesize that NP exposure promotes the growth of *Ruminococcaceae* sp. by modulating goblet-like cell function, posing potential risks to the host.

(5) Conclusion

NP alters the mucus-microbiota interaction by selectively impacting certain bacterial species, such as *Lachnospiraceae* and *Ruminococcaceae*. This is mediated through changes in host microRNAs and EV production, highlighting the complex pathways by which NP disrupts the intestinal environment.

We believe this explanation clarifies the mechanisms underlying the observed effects and addresses the reviewer's concerns comprehensively.

The authors should examine the level of EVs in the intestinal contents to better conclude that NP is capable of altering EVs. The in vitro experiments revealed that NP changes EVs. The authors should also demonstrate in vivo that the EVs of these bacteria also have effects on the host's gut function or microenvironment.

Ans: We appreciate the reviewer's insightful comment. To address this issue, we measured and validated the levels of EVs in the intestinal contents.

As described in the revised manuscript (lines 349-356), PS-NP treatment was found to increase EV concentrations in both the host and intestinal microbiota. Specifically, EV derived from goblet-like LS174T cells were shown to promote the proliferation of *Ruminococcaceae* sp., contributing to gut microbiota imbalance.

To further confirm the impact of NP on EV levels within the gut lumen, we quantitatively measured EV concentrations in culture media derived from mouse intestinal tissue and fecal microbiota cultures, both with and without NP treatment.

These results are presented in **Supplementary Fig. 9**.

We believe these additional data provide strong evidence for the role of NP in altering EV levels and their subsequent effects on the gut microenvironment.

We would like to express our sincere gratitude to the Editor and Reviewers for their insightful comments and constructive feedback on our manuscript. We have carefully considered each suggestion and revised the manuscript accordingly, as detailed in our responses below. We appreciate the time and effort invested in reviewing our work and hope that the improvements we have made will meet the standards of *Nature Communications.*

Reviewer #1 (Remarks to the Author):

Given the potential risk of nanopalastics (NP) on human health, this study is both intriguing and signficant to understand the mechanisms by which NP nanoparticles impact the gut barrier integrity and gut microbiota. Hsu and coauthors demonstrate here that NP nanoparticles interfer with tight junction proteins expression via their effect on the host intestinal and EV microRNAs.This NP-induced alteration in EV content of microRNAs in turn induces gut microbiota dysbiosis. Also, EV of some microbiota members exposed to NP suppress MUC-13 expression. The study is signficant to the field and the authors have addressed my concerns raised in the first revision, So I recommend accepting the manuscript for publication in its current format.

Ans: We sincerely appreciate the reviewer's positive evaluation and insightful comments. We are grateful for the recognition of the significance of our study and its contribution to understanding the impact of nanoplastics (NP) on gut barrier integrity and microbiota dysbiosis via extracellular vesicle (EV)-mediated microRNA regulation.

Thank you for acknowledging that we have satisfactorily addressed the concerns raised in the previous revision. We are pleased that the manuscript is now considered suitable for publication in its current format.

We are confident that the findings presented in our study will provide valuable insights into the potential health risks associated with NP exposure and contribute to future research in this field.

Thank you again for your thoughtful review and recommendation for acceptance.