Article:
Wu, D., Carter, L., Kay, P. et al. (3 more authors) (2025) Female zebrafish are more affected than males under polystyrene microplastics exposure. Journal of Hazardous Materials, 482. 136616. ISSN 0304-3894

https://doi.org/10.1016/j.jhazmat.2024.136616

Female zebrafish are more affected than males under polystyrene microplastics exposure

Di Wu[1, 2], Laura Carter[2], Paul Kay[2], Joseph Holden[2], Ying Yin[1]*, Hongyan Guo[1]

1 State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing, 210023, China

2 water@leeds, School of Geography, University of Leeds, Leeds LS2 9JT, UK

*Corresponding author. Tel.: +86-25-89680263; Fax: +86-25-89680263.

E-mail address: yinying@nju.edu.cn (Yin Y).

Table S1 Primers of the selected genes in this study

Primer pairs	Sequence (5'—3')
341F	CCTAYGGGRBGCASCAG
806R	GGACTACNNGGGTATCTAAT

Table S2 Shannon and Simpson alpha diversity index of zebrafish gut microbiota after PS microplastics exposure (n = 4).

Index	Shannon	Simpson
♀ck	2.514 ± 0.771	0.559 ± 0.123
♀1μm	4.663 ± 0.899*	0.872 ± 0.096*
♂ck	4.508 ± 0.966	0.857 ± 0.062
♂1μm	8.222 ± 0.282*	0.987 ± 0.002*

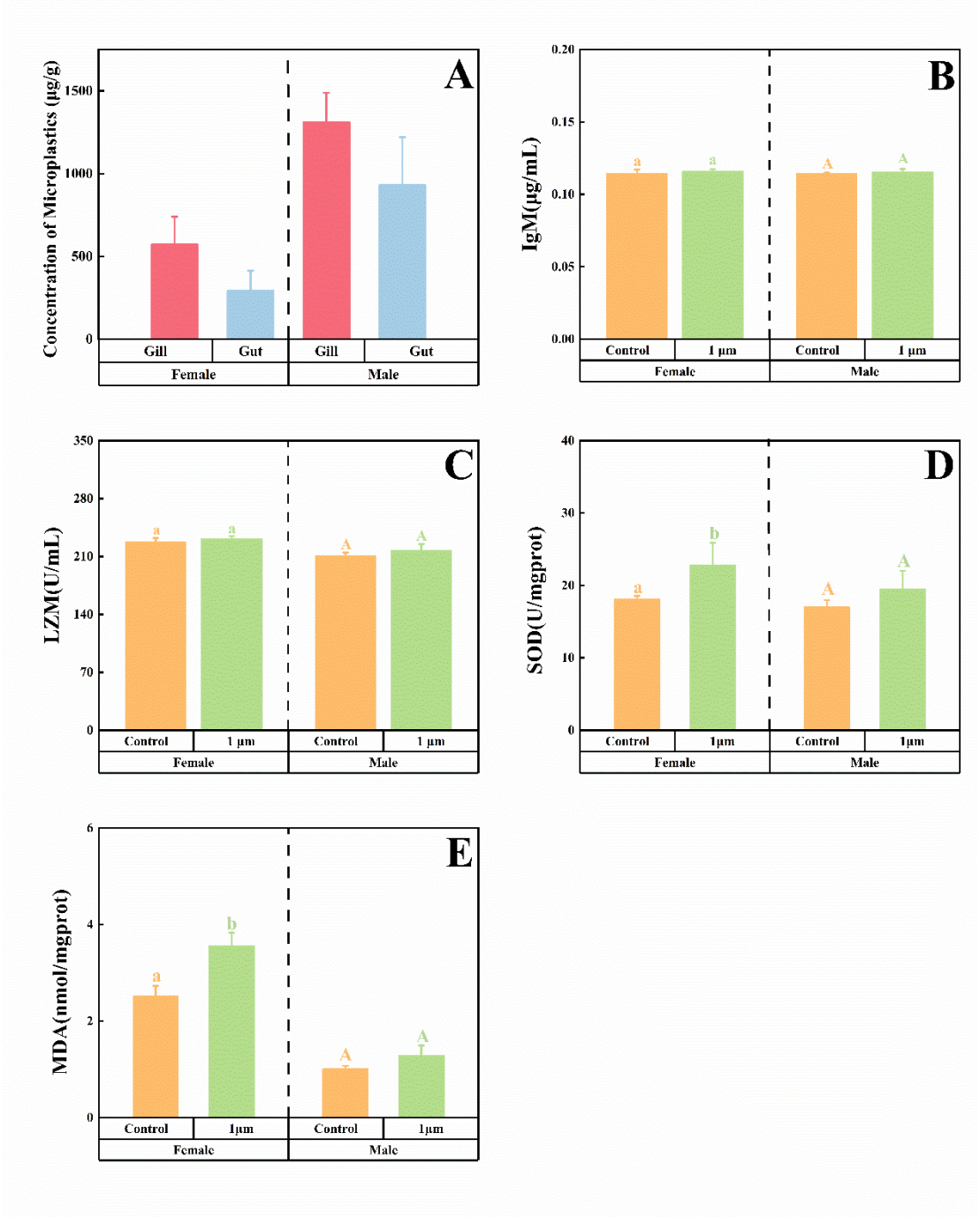

Figure S1. Effects of PS microplastics exposure on microplastics uptake, skin mucus and oxidative stress of zebrafish. (A) Accumulation of PS microplastics in the gill and gut of zebrafish after exposure for 21 days. (B) Changes in immunologic factors of zebrafish skin mucus after exposure to PS microplastics: IgM; (C) LZM. (D) Anti-oxidative enzyme activities of zebrafish after exposure to PS microplastics: SOD; (E)

MDA. Data represent mean ± SD (n = 4). Different letters indicate significant differences between treatments ($p < 0.05$).

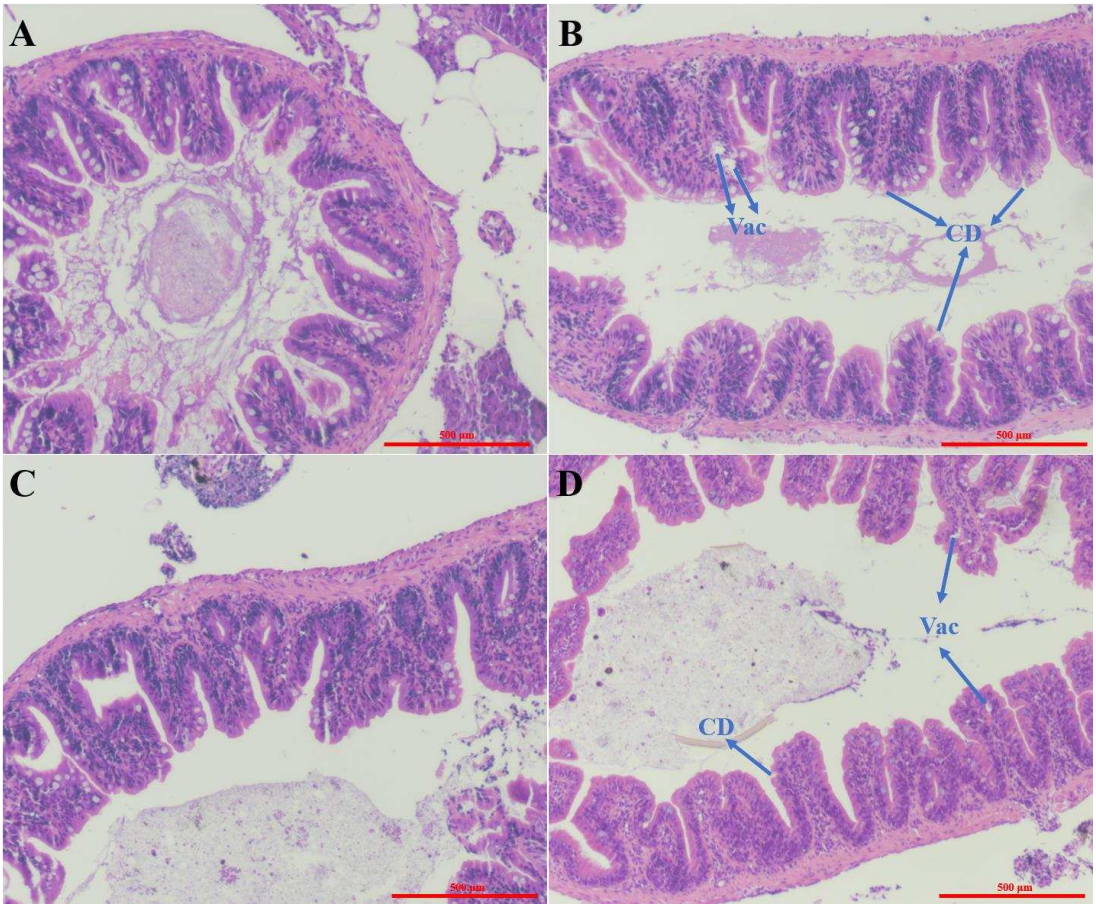

Figure S2. Histological damage of zebrafish gut caused by PS microplastics exposure. (A) control of female zebrafish; (B) PS microplastics exposure of female zebrafish; (C) control of male zebrafish; (D) PS microplastics exposure of male zebrafish. Histological changes including vacuolization (Vac) and cilia defects (CD) are marked.

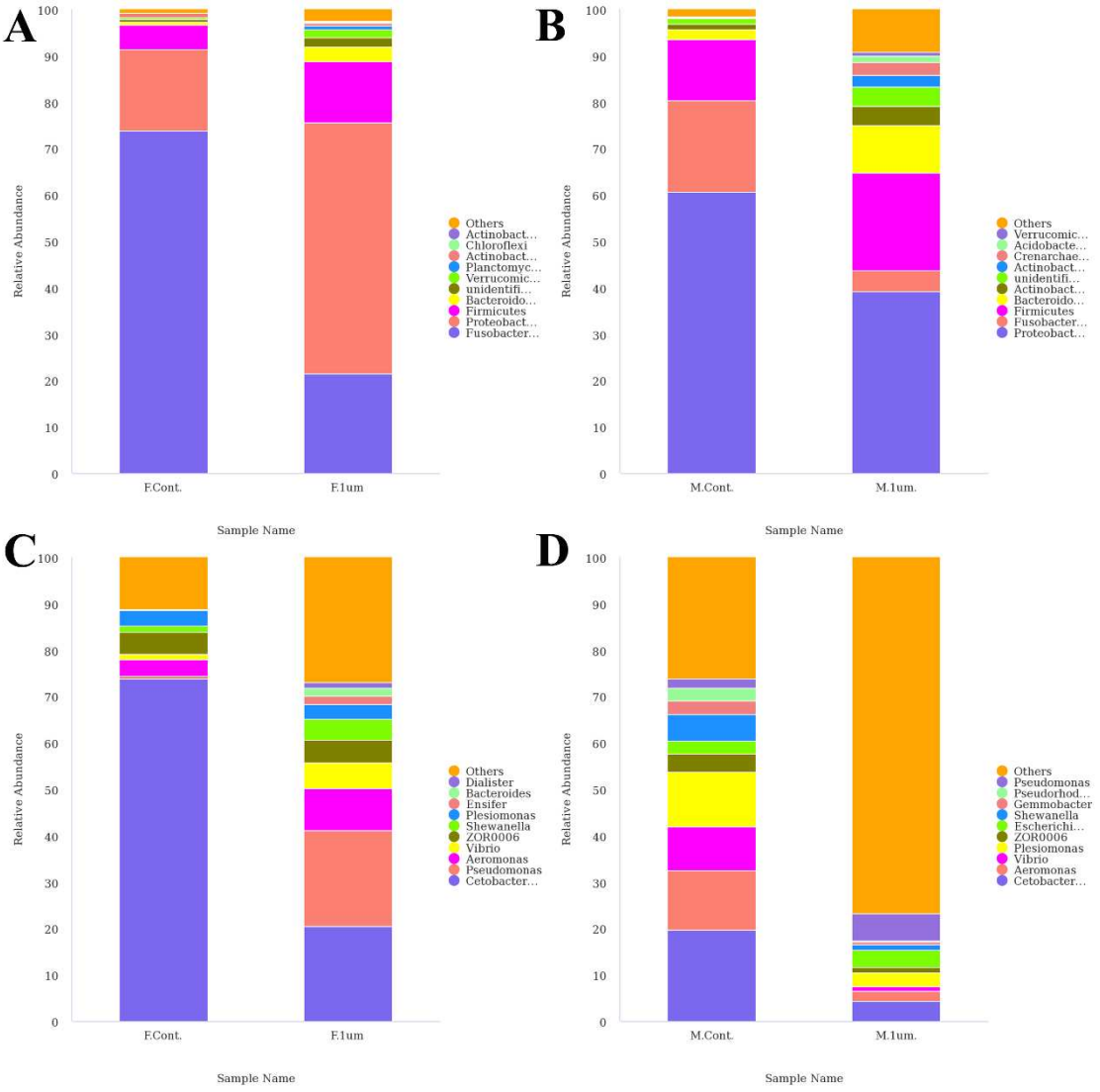

Figure S3. The relative abundance of gut microbiota at phylum level after PS microplastics exposure: (A) female groups; (B) male groups. The relative abundance of gut microbiota at genus level after PS microplastics exposure: (C) female groups; (D) male groups.

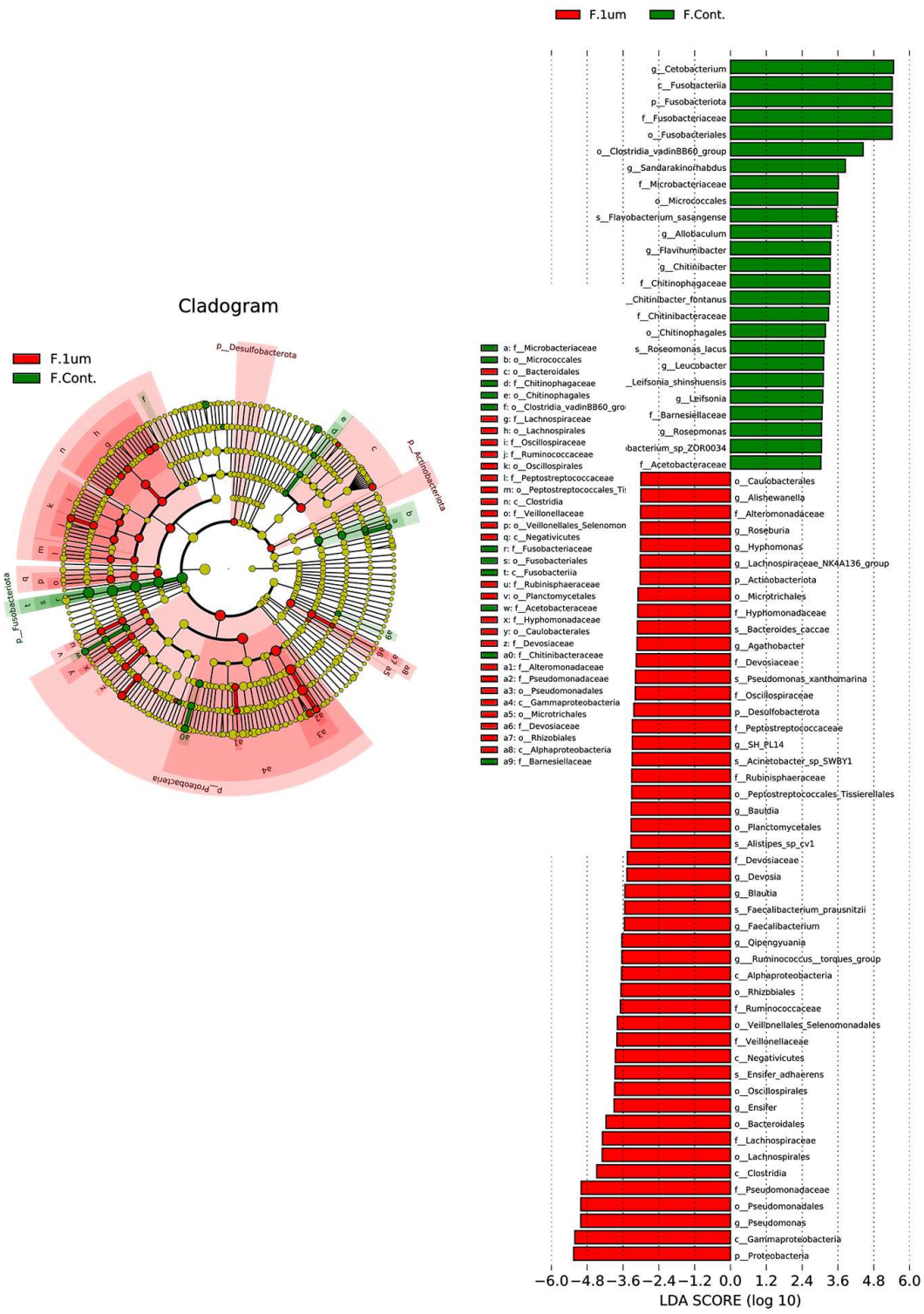

Figure S4. LEfSe analysis of gut bacteria of female zebrafish affected by PS microplastics. The circles in the cladogram represent taxa from kingdom to genus (from inside to outside).

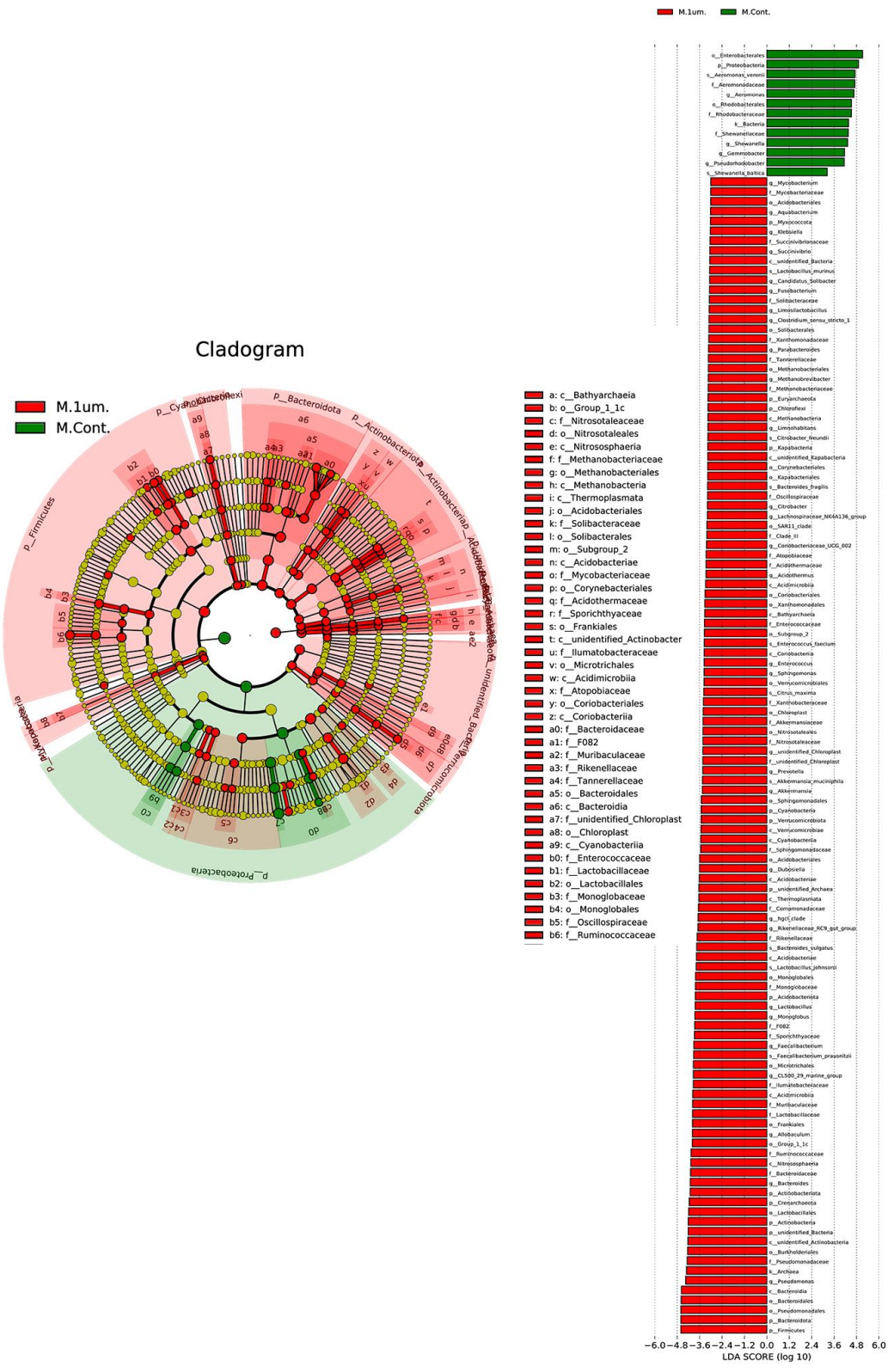

Figure S5. LEfSe analysis of gut bacteria of male zebrafish affected by PS microplastics. The circles in the cladogram represent taxa from kingdom to genus (from inside to outside).

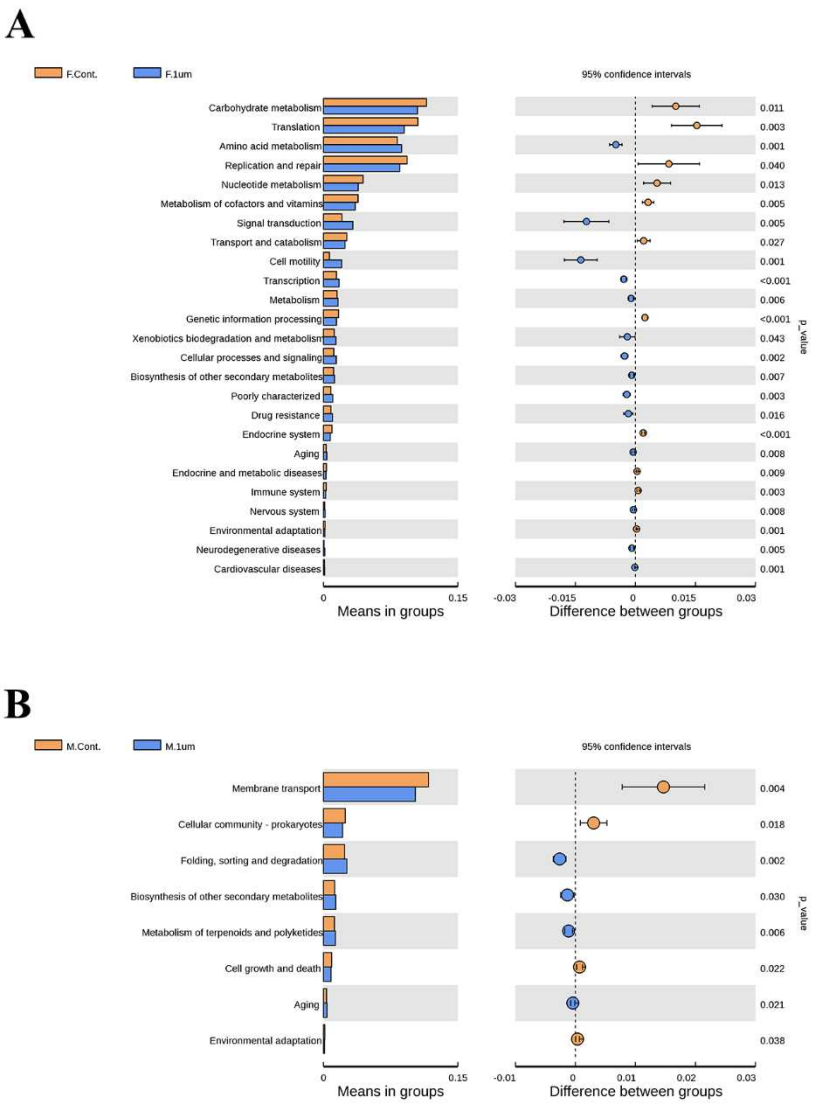

Figure S6. Welch's t-test analysis of predicted function (level 2) of zebrafish gut microbiota by Tax4Fun analysis: (A) female groups; (B) male groups.

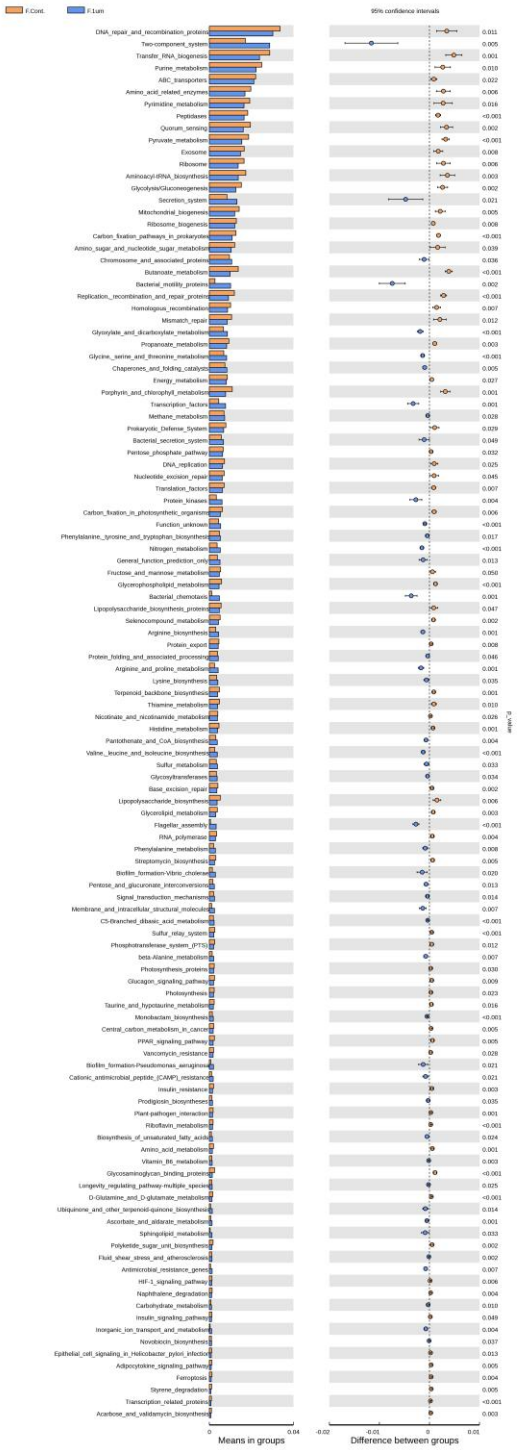

Figure S7. Welch's t-test analysis of predicted function (level 3) of zebrafish gut microbiota in female groups by Tax4Fun analysis.

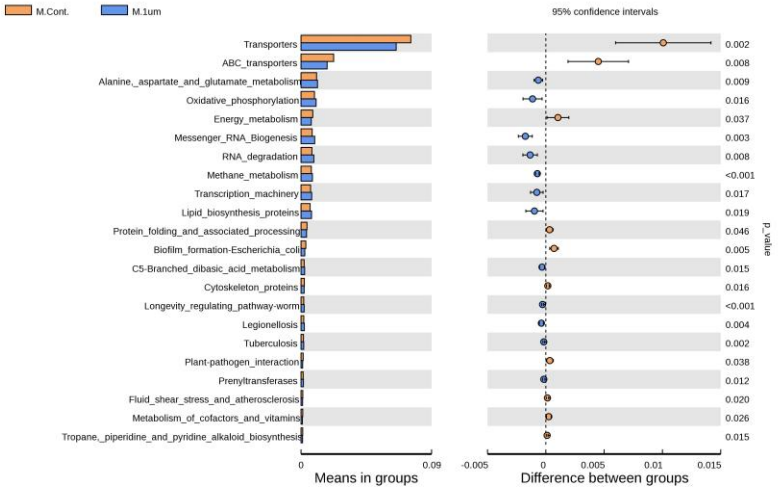

Figure S8. Welch's t-test analysis of predicted function (level 3) of zebrafish gut microbiota in male groups by Tax4Fun analysis.

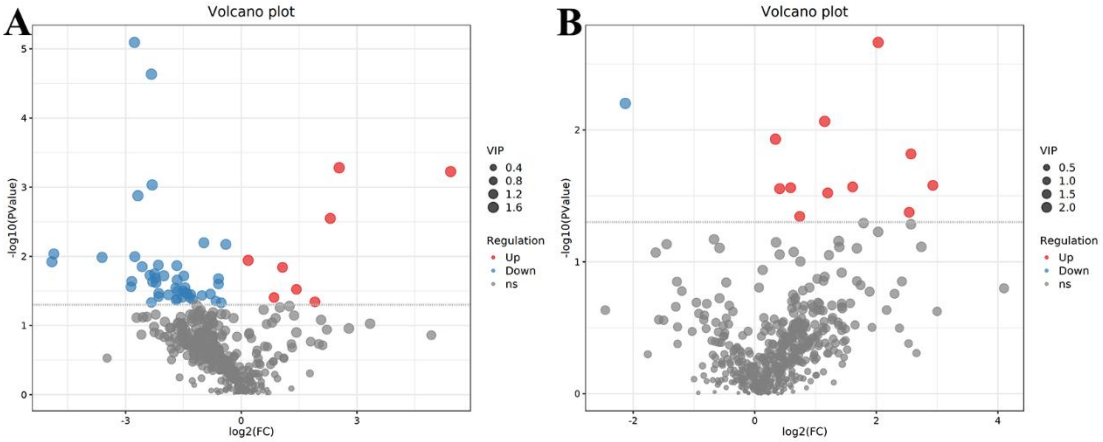

Figure S9. Volcano plot of differential metabolites after PS microplastics exposure: (A) female group; (B) male group.

Text S1. 16S rRNA sequencing

Extraction of genome DNA: The CTAB/SDS method was used to extract the total genome DNA in samples. DNA concentration and purity were monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1ng/μL with sterile water.

Amplicon Generation: 16S rRNA genes in distinct regions (16S V3-V4) were amplified with specific primer and barcodes. Primer sequences are shown in Table S1 [1]. All PCR mixtures contained 15 μL of Phusion® High-fidelity PCR Master Mix (New England Biolabs), 1 μM of each primer and 10ng target DNA. Cycling conditions consisted of a first denaturation step at 98°C for 1 min, followed by 30 cycles at 98°C (10s), 50°C(30s) and 72°C(30s) and a final 5 min extension at 72°C.

PCR Products quantification and qualification: An equal volume of 1X loading buffer (contained SYBR green) was mixed with PCR products and electrophoresis was performed on 2% agarose gel for DNA detection. The PCR products were mixed in equal proportions, and then Universal DNA PCR Purification Kit (TianGen, China, Catalog #: DP214) was used to purify the mixed PCR products.

Library preparation and sequencing: Following the manufacturer's recommendations, sequencing libraries were generated with NEB Next Ultra DNA Library Prep Kit for Illumina (NEB, USA, Catalog #: E7370L). The library quality was assessed on the Agilent 5400 system (Agilent, USA) and quantified by real-time PCR (1.5nM). Finally, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated.

Data analysis: The raw reads were merged using FLASH (V1.2.7) and then filtered

to remove low-quality reads and chimera sequences using QIIME (v1.9.1) and UCHIME. The effective tags were then clustered by UPARSE to Operational Taxonomic Units (OTUs) according to 97% similarity. For each representative sequence, the Silva Database was used based on Mothur algorithm to annotate taxonomic information and count the community composition of classification level (e.g., phylum and genus). Alpha diversity and beta diversity were calculated with QIIME. Gut microbiota functions were predicted using Tax4Fun, and pathway information were performed on Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/).

Text S2. Liver metabolomics

Sample preparation

Sample was accurately weighed into a 2 mL centrifuge tube with 1000 μL tissue extract (75% 9:1 methanol: chloroform, 25% H2O) and 3 steel balls. Then the sample was ground at 50 Hz for 60 s in tissue grinder twice. After grinding, sample was sonicated for 30 min and cooled in an ice bath for 30 min. The sample was centrifuged at 12,000 rpm and 4 °C for 10 min. The supernatant was transferred to a new 2 mL centrifuge tube, vacuum condensed and dried for 9 hours (Eppendorf Concentrator plus). 200 μL 50% acetonitrile solution prepared with 2-Amino-3-(2-chloro- phenyl)-propionic acid (4 ppm) (stored at 4 ℃) was added to re dissolve the sample. The supernatant was filtered by 0.22 μm polytetrafluoroethylene (PTFE) membrane and transferred into the detection bottle for LC-MS detection.

Liquid chromatography conditions

The LC analysis was performed on a Vanquish UHPLC System (Thermo Fisher Scientific, USA). Chromatography was carried out with an ACQUITY UPLC ® HSS T3 (150×2.1 mm, 1.8 μm) (Waters, Milford, MA, USA). The column maintained at 40 ℃. The flow rate and injection volume were set at 0.25 mL/min and 2 μL, respectively. For LC-ESI (+)-MS analysis, the mobile phases consisted of (A) 0.1% formic acid in acetonitrile (v/v) and (B) 0.1% formic acid in water (v/v). Separation was conducted under the following gradient: 0~1 min, 2% A; 1~9 min, 2%~50% A; 9~12 min, 50%~98% A 12~13.5 min, 98% A; 13.5~14 min, 98%~2% A; 14~20 min, 2% A. For LC-ESI (-)-MS analysis, the analytes were carried out with (C) acetonitrile and (D) ammonium formate (5mM). Separation was conducted under the following gradient: 0~1 min, 2% C; 1~9 min, 2%~50% C; 9~12 min, 50%~98% C; 12~13.5 min, 98% C; 13.5~14 min, 98%~2% C; 14~17 min, 2% C.

Mass spectrum conditions

Mass spectrometric detection of metabolites was performed on Orbitrap Exploris 120 (Thermo Fisher Scientific, USA) with ESI ion source. Simultaneous MS1 and MS/MS (Full MS-ddMS2 mode, data-dependent MS/MS) acquisition was used. The parameters were as follows: sheath gas pressure, 30 arb; aux gas flow, 10 arb; spray voltage, 3.50 kV and -2.50 kV for ESI (+) and ESI (-), respectively; capillary temperature, 325 ℃; MS1 range, m/z 100-1000; MS1 resolving power, 60000 FWHM; number of data dependent scans per cycle, 4; MS/MS resolving power, 15000 FWHM; normalized collision energy, 30%; dynamic exclusion time, automatic.

Data processing and multivariate analysis

The raw data were firstly converted to mzXML format by MSConvert in the ProteoWizard software package (v3.0.8789) and processed using XCMS for feature detection, retention time correction and alignment. The metabolites were identified by accuracy mass (< 30 ppm) and MS/MS data which were matched with HMDB (http://www.hmdb.ca), massbank (http://www.massbank.jp/), LipidMaps (http://www.lipidmaps.org), mzcloud (https://www.mzcloud.org) and KEGG (http://www.genome.jp/kegg/). The robust LOESS signal correction (QC-RLSC) was applied for data normalization to correct for any systematic bias. After normalization, only ion peaks with relative standard deviations (RSDs) less than 30 % in QC were kept to ensure proper metabolite identification.

The Ropls software was used for all multivariate data analyses and modeling. Data were mean-centered using scaling. Models were built on principal component analysis (PCA), orthogonal partial least-square discriminant analysis (PLS-DA) and partial least-square discriminant analysis (OPLS-DA). The metabolic profiles could be visualized as a score plot, where each point represents a sample. The corresponding loading plot and S-plot were generated to provide information on the metabolites that influence clustering of the samples. All of the models evaluated were tested for over fitting with methods of permutation tests. The descriptive performance of the models was determined by R2X (cumulative) (perfect model: R2X (cum) = 1) and R2Y (cumulative) (perfect model: R2Y (cum) = 1) values while their prediction performance was measured by Q2 (cumulative) (perfect model: Q2 (cum) = 1) and a permutation test. The permuted model should not be able to predict classes: R2 and Q2 values at the Y-

axis intercept must be lower than those of Q2 and the R2 of the non-permuted model. OPLS-DA allowed the determination of discriminating metabolites using the variable importance on projection (VIP). The variable importance projection (VIP) produced by OPLS-DA fold change (FC), was applied to discover the contributable-variable for classification. Finally, P values $<$ 0.05 and VIP values $>$ 1 were considered to be statistically significant metabolites.

Pathway analysis

Differential metabolites were subjected to pathway analysis by MetaboAnalyst, which combines results from powerful pathway enrichment analysis with the pathway topology analysis. The identified metabolites in metabolomics were then mapped to the KEGG pathway for biological interpretation of higher-level systemic functions. The metabolites and corresponding pathways were visualized using KEGG Mapper tool.

Reference

[1] M. Qian, J. Wang, X. Ji, H. Yang, B. Tang, H. Zhang, G. Yang, Z. Bao, Y. Jin, Sub-chronic exposure to antibiotics doxycycline, oxytetracycline or florfenicol impacts gut barrier and induces gut microbiota dysbiosis in adult zebrafish (Daino rerio), Ecotoxicology and Environmental Safety, 221 (2021) 112464.