Supporting Information

Sub-chronic exposure to ambient ozone induced liver and lung damage: revealed by lung-liver axis

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LISTS

SUPPORTING TEXT

Text S1. The 16S rRNA gene sequencing and analysis of lung microbiome

DNA extraction, PCR, and Illumina Nextseq 2000 sequencing

Genomic DNA from individual lung samples were extracted using the NEBNext Microbiome DNA Enrichment Kit (Omega Bio-tek, Norcross, GA, U.S.) according to the manufacturer's instructions. The quality and concentration of DNA were determined by 1.0% agarose gel electrophoresis and a NanoDrop2000 spectrophotometer (Thermo Scientific, United States) and kept at -80 ℃ prior to further use. The hypervariable region V3-V4 of the bacterial 16S rRNA gene were amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R(5'-GGACTACHVGGGTWTCTAAT-3')[1] by T100 Thermal Cycler PCR thermocycler (BIO-RAD, USA). The PCR reaction mixture including 4 μL 5 × Fast Pfu buffer, 2 μL 2.5 mM dNTPs, 0.8 μL each primer (5 μM), 0.4 μL Fast Pfu polymerase, 10 ng of template DNA, and ddH2O to a final volume of 20 μL. PCR amplification cycling conditions were as follows: initial denaturation at 95 ℃ for 3 min, followed by 27 cycles of denaturing at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃for 45 s, and single extension at 72 ℃ for 10 min, and end at 4 ℃. The PCR product was extracted from 2% agarose gel and purified using the PCR Clean-Up Kit (YuHua, Shanghai, China) according to the manufacturer's instructions and quantified using Qubit 4.0 (Thermo Fisher Scientific, USA).

Illumina PE300/PE250 sequencing

Purified amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina PE300 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw sequencing reads were deposited into the NCBI Sequence Read Archive (SRA) database.

Sequence data analysis

Quality filtering on the paired-end raw reads were performed under specific filtering conditions to obtain the high-quality clean reads according to the

Trimmomatic (V0.33, http://www.usadellab.org/cms/?page=trimmomatic) quality controlled process. Paired-end clean reads were merged using FLASH (V1.2.11, https://ccb.jhu.edu/software/FLASH/) according to the relationship of the overlap between the paired-end reads, when at least 10 of the reads overlap the read generated from the opposite end of the same DNA fragment, the maximum allowable error ratio of the overlap region of 0.1, and the spliced sequences were called Raw Tags. Quality filtering, length trimming, and homopolymer truncation were processed using Mothur software (V1.35.1, http://www.mothur.org). Sequence analysis was performed by usearch software (V10, http://www.drive5.com/usearch/). Sequences with ≥97% similarity were assigned to the same operational taxonomic unit (OTU). The most frequently occurring sequence was extracted as the representative sequence for each OTU and was screened for further annotation using Silva (for 16S, https://www.arb-silva.de/) and Unite (for ITS, http://unite.ut.ee/index.php) databases with the confidence threshold set to default to ≥0.5. The OTU taxonomy synthesis information table (out_table) for the final analysis was obtained after removing the OTUs and tags, which are annotated as chloroplasts or mitochondria (16S amplicons), or organisms other than fungi (ITS amplicons), and could not be annotated to the kingdom level.

Statistical analysis

Alpha diversity was calculated using QIIME2-2021.2. R software (version R-4.0.2) was used to conduct nonparametric tests on the Alpha diversity index with EasyStat package (wilcoxon. test, $P<0.05$ indicates a significant difference). Different letter marks indicate significant differences. Beta diversity was calculated by QIIME2-2021.2. Principal coordinate analysis (PCoA) and visualization were conducted by ggplot2 package based on abund_jaccard distance. Non-parametric multivariate statistical tests were performed by R(4.0.2) based on vegan package: multivariate analysis of variance based on Bray_Curtis distance (Analysis of variance, ADONIS), variation analysis between groups (Analysis of similarities, ANOSIM), multiple response permutation procedure (MRPP).Different species detection between groups: one-way ANOVA test analysis was used to find the biomarker

between groups.

Lung Microbiome Health Index (LMHI)

The Lung Microbiome Health Index (LMHI), a robust index that assesses health status (i.e., the presence of diagnosed disease) based on species-level taxonomic characteristics of lung microbiome samples, focusing on determining the likelihood of disease, independent of clinical diagnosis; This is done by comparing the relative abundance of two groups of microbial species that are associated with good and poor health[1].

Microbiome Dysbiosis Index (MDI)

The Microbiome Dysbiosis Index (MDI) is an index that determines the degree of microbial imbalance. The higher the value, the greater the degree of bacterial disturbance[2].

Text S2. The 16S rRNA gene sequencing and analysis of lung microbiome

For the sample preparation (n = 6), methods were conducted by a previous study3. Briefly, a liver sample (100 mg) was homogenized in 225 μL -20°C methanol containing L-methionine-methyl-d3 internal standard using a GenoGrinder 2010 (SPEX SamplePrep) at 1,350 rpm for 3 min, followed by sequential addition of 750 μL MTBE (with internal standards), vortexing (30 s), 4°C agitation (5 min), and 188 μL water to induce phase separation after additional vortexing (30 s). After centrifugation (14,000 g, 2 min), 350 μ L upper (non-polar) and 250 μL lower (polar) phases were collected and dried, with remaining fractions pooled as Quality control (QC) samples that were injected after every 5 biological samples.

The raw mass spectrometry data were processed through a comprehensive preprocessing pipeline involving peak detection, retention time alignment, and intensity quantification, from which key metabolite features including retention times, compound identities, and peak areas were extracted before being imported into R software (version 4.4.1) for subsequent statistical analysis, with data normalization conducted using internal standards to account for systematic variations, while QC samples were utilized for signal drift correction through the robust spline correction (RSC) algorithm, followed by missing value imputation using the Random Forest method to maintain data integrity and log2 transformation to achieve variance stabilization and approximate normality, ultimately enabling the identification of differentially expressed metabolites between experimental groups via orthogonal partial least squares-discriminant analysis (OPLS-DA) with stringent selection criteria of variable importance in projection (VIP) scores >1 and statistically significant P-values <0.05 from Student's t-test.

SUPPORTING FIGURE

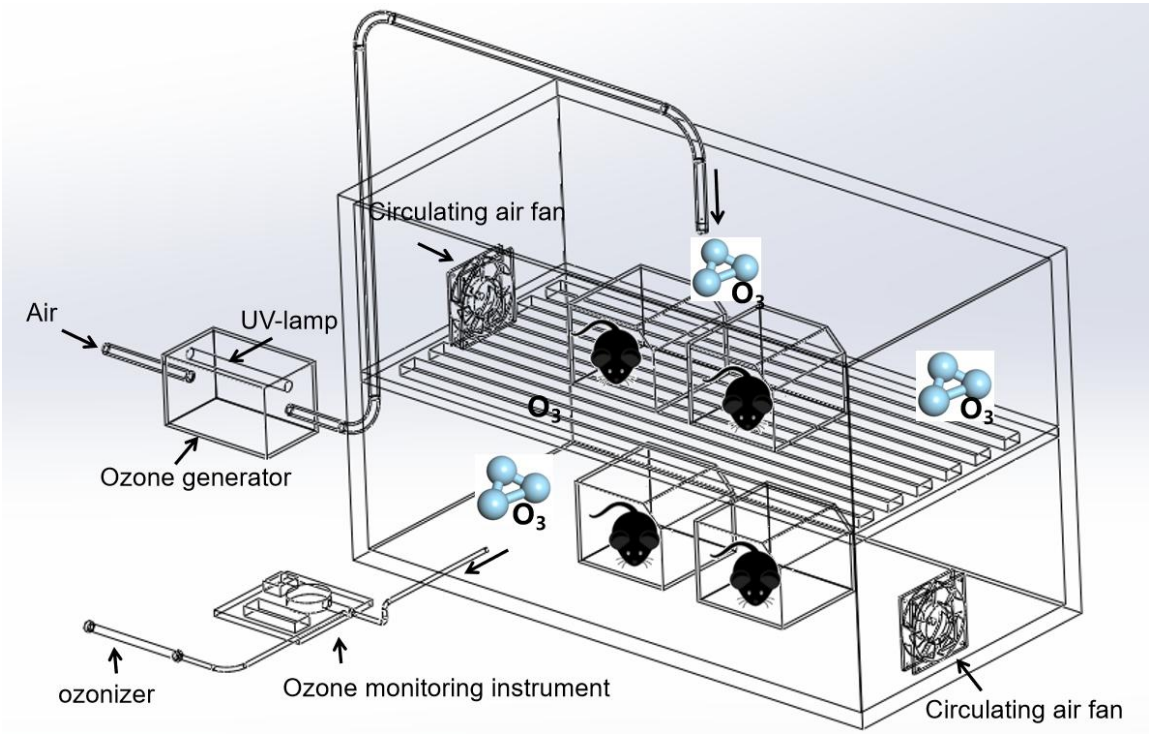

Fig. S1. The O_3 exposure system of the study.

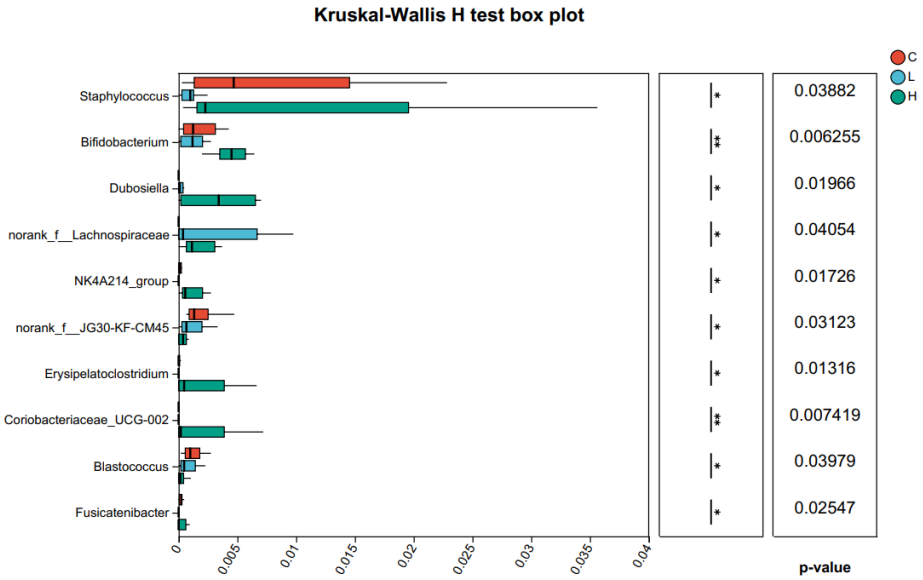

Fig. S2. Differential lung bacteria in genera level of different exposure groups. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; multiple group comparisons were performed using Kruskal-Wallis test. The C, L, and H represent the control group, low (320 μg/m^3) and high (1280 μg/m^3) groups, respectively.

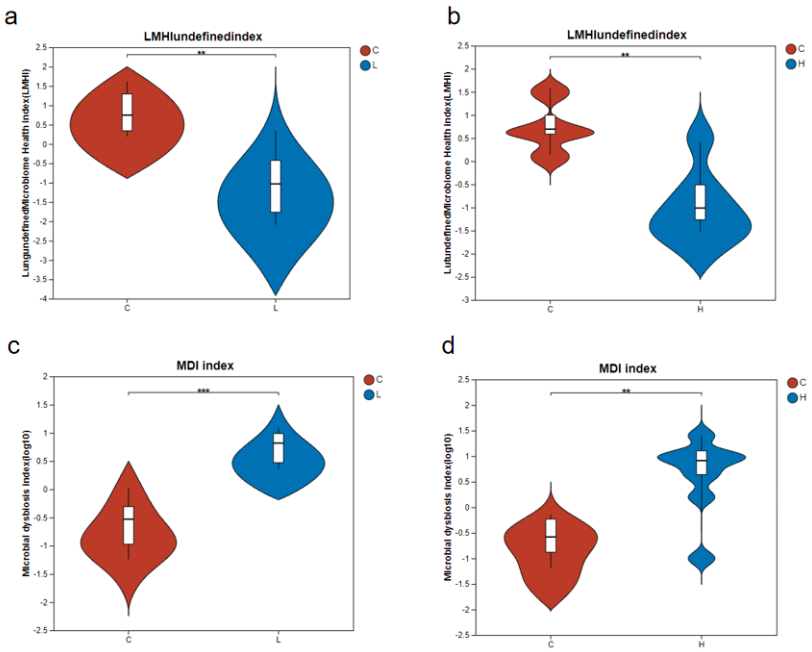

Fig. S3. The Lung Microbiome Health Index and Microbiome Dysbiosis Index analysis. (a-b) represents the LMHI index in the fecal of different groups. **(c-d)** represents MDI index in lung of different groups. The C, L, and H represent the control group, mice exposed to low (320 μg/m^3) and high (1280 μg/m^3) concentrations of ozone groups respectively. Difference group comparisons were performed using the student *t-test*, **P* < 0.05.

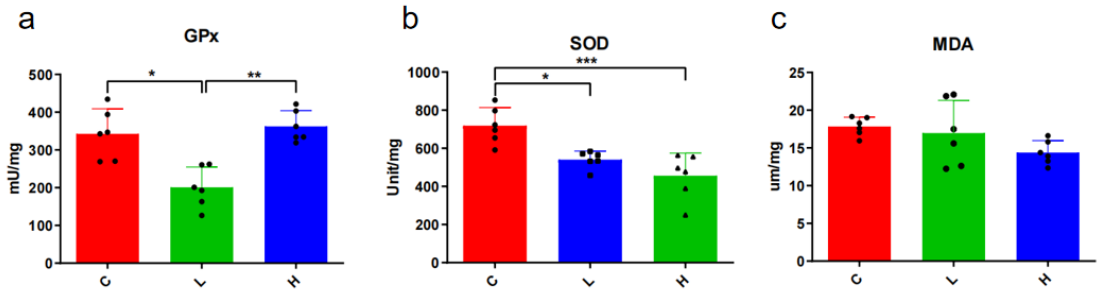

Fig. S4. Levels of oxidative stress indicators in lung tissues of different groups (*n* = 6). The C, L, and H represent the control group, mice exposed to low (320 μg/m^3) and high (1280 μg/m^3) concentrations of ozone groups respectively. multiple group comparisons were performed using a one-way ANOVA test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

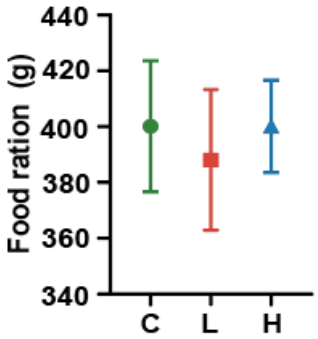

Fig. S5. Food consumption throughout the animal experiment. The C, L, and H represent the control group, mice exposed to low (320 μg/m^3) and high (1280 μg/m^3) concentrations of ozone groups respectively.

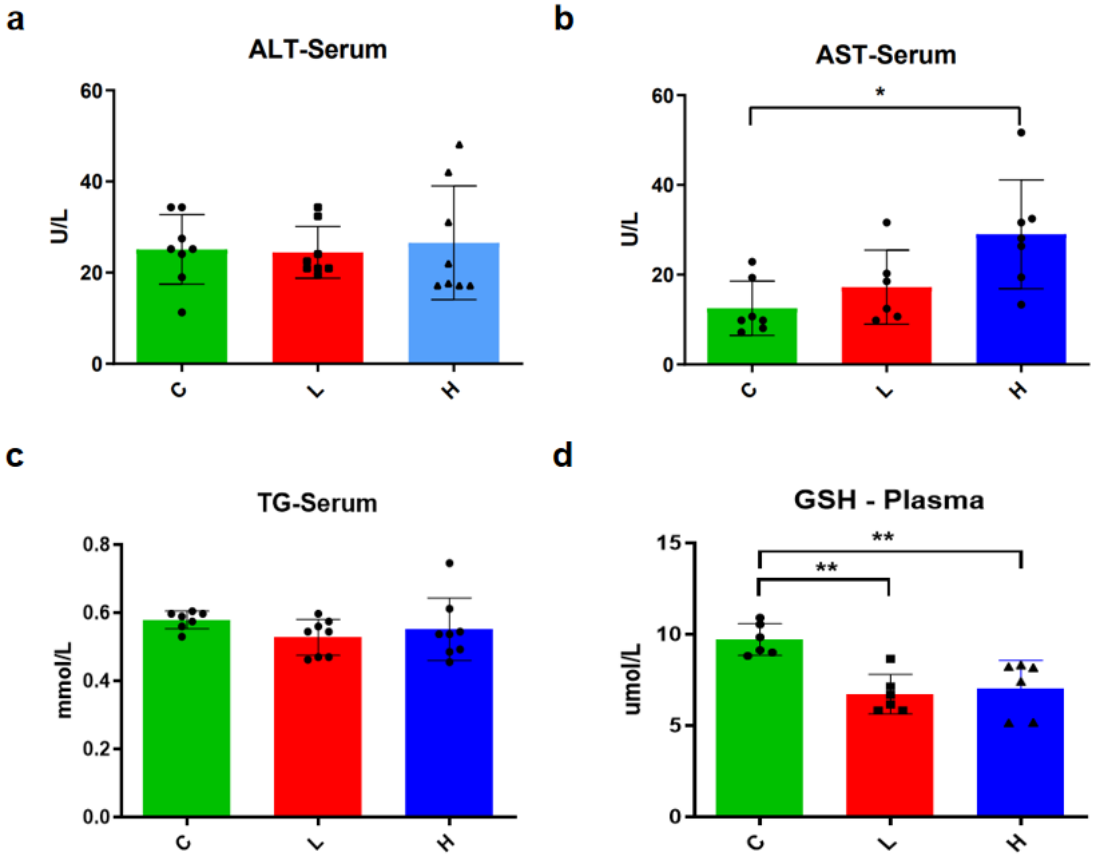

Fig. S6. Levels of liver functional in serun and GSH in plasma of different groups (*n* = 6-8). The C, L, and H represent the control group, mice exposed to low (320 μg/m^3) and high (1280 μg/m^3) concentrations of ozone groups respectively. multiple group comparisons were performed using a one-way ANOVA test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

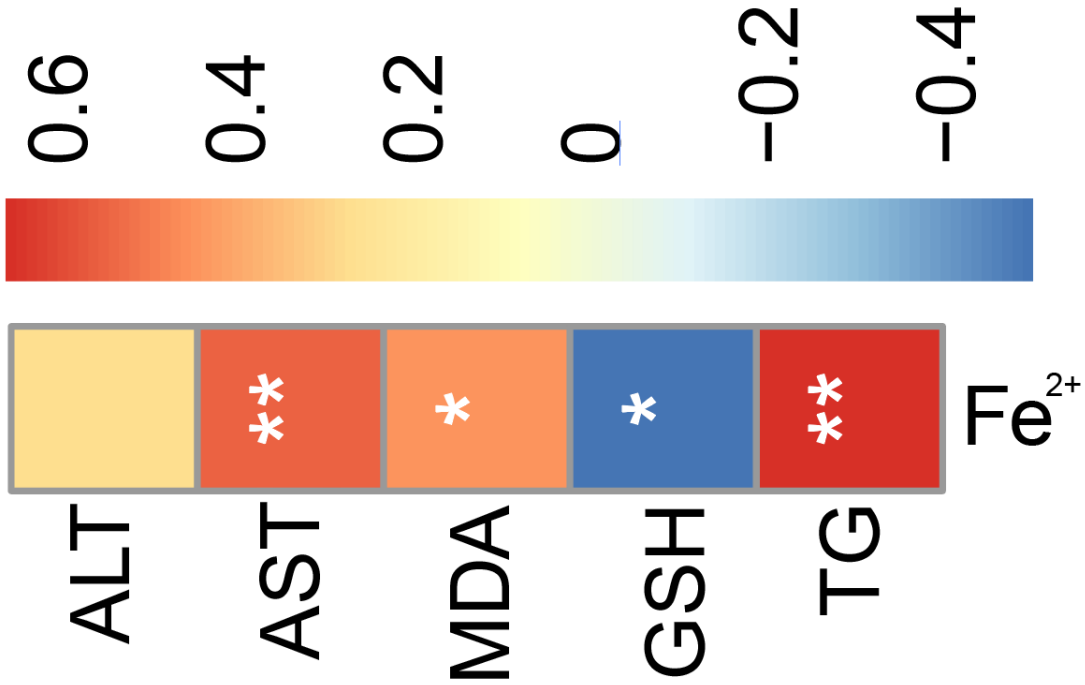

Fig. S7. Spearman's correlation analyses between Fe^{2+} with ALT, AST, MDA, GSH and TG of all groups. $^{*}p < 0.05$, $^{**}p < 0.01$.

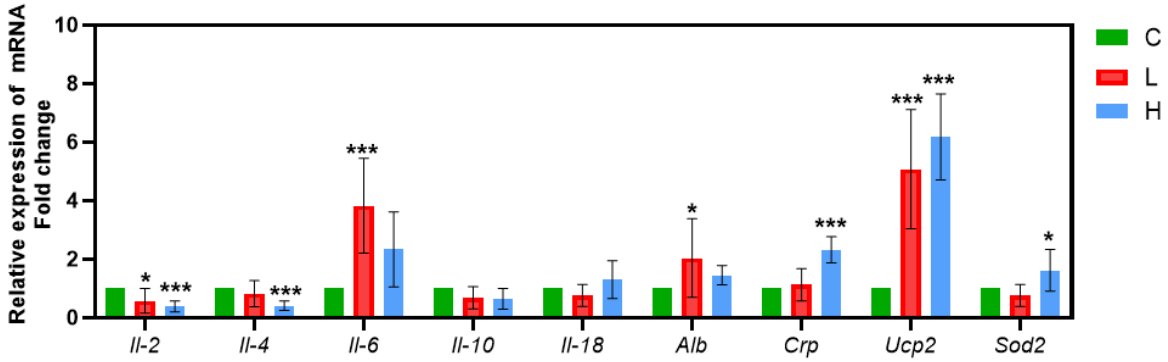

Fig. S8. The relative expression levels of 9 functional genes in the liver (*n* = 6-8). The C, L, and H represent the control group, mice exposed to low (320 μg/m^3) and high (1280 μg/m^3) concentrations of ozone groups respectively. multiple group comparisons were performed using a one-way ANOVA test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

SUPPORTING TABLE

Table S1. Nucleotide Sequences of the qPCR Primers.

Gene	Sequence (5'->3')
Gapdh	AGGTCGGTGTGAACGGATTTG
	GGGGTCGTTGATGGCAACA
Occludin	TGAACAGCCCCCCAATGT
	TCAACTCTTTCCGCATAGTCAGAT
Zo-1	GCTTTAGCGAACAGAAGGAGC
	TTCATTTTTCCGAGACTTCACCA
Hmox-1	AGGGCTCGGAACTCCAGAAA
	CCAGGGAATCGGTAGACATCG
Sod2	GTTACAACTCAGGTCGCTCTTCAG
	TGATAGCCTCCAGCAACTCTCC
Ucp2	GCTGGTGGTGGTCGGAGATAC
	GCACAGTTGACAATGGCATTACG
Nfe2l2	AAGCACAGCCAGCACATTCTC
	ACCAGGACTCACGGGAACTTC
Nos3	GCCTAGTCCTCGCCTCCTTC
	TGAACCACTTCCATTCTTCGTAGC
Cat	CGTCCGTCCCTGCTGTCTC
	GCTCCTTCCACTGCTTCATCTG
Gstp1	GCTGGAAGGAGGAGGTGGTTAC
	AGGTGTCTCAAGATGGCATTAGATTG
Il-1β	TCGCTCAGGGTCACAAGAAA
	CATCAGAGGCAAGGAGGAAAAC
Il-2	TGAGCAGGATGGAGAATTACAGG
	GTCCAAGTTCATCTTCTAGGCAC
Il-4	GGTCTCAACCCCCAGCTAGT

	GCCGATGATCTCTCTCAAGTGAT

Gene	Sequence (5'->3')
Il-6	CTGCAAGAGACTTCCATCCAGTT
	GAAGTAGGGAAGGCCGTGG
Il-10	GCTCTTACTGACTGGCATGAG
	CGCAGCTCTAGGAGCATGTG
Il-18	GACTCTTGCGTCAACTTCAAGG
	CAGGCTGTCTTTTGTCAACGA
Tlr-4	GCCTTTCAGGGAATTAAGCTCC
	GATCAACCGATGGACGTGTAAA
Mmp9	GCTGACTACGATAAGGACGGCA
	TAGTGGTGCAGGCAGAGTAGGA
Crp	TTCCCAAGGAGTCAGATACTTCC
	TCAGAGCAGTGTAGAAATGGAGA
Alb	GACGTGTGTTGCCGATGAGT
	GTTTTCACGGAGGTTTGGAATG

Table S2. Differential comparison of lung microbial community structure.

Adonis	Df	Sums_of_sqs	F.Model	R^2	*Pr* (>F)
Group factors	2	0.7995	1.6751	0.1376	0.05
Residuals	21	5.0115	-	0.8624	0
Total	23	5.811	-	1	0

Note: The C, L, and H represent the control group, mice exposed to low (320 μg/m^3) and high (1280 μg/m^3) concentrations of ozone groups respectively.

Adonis: F represents F test value, and R^2 represents the interpretation degree of sample difference by different groups. The larger F and R2 are, the higher the degree of group difference is. $P < 0.05$ indicates that the test has a high feasibility.

REFERENCES

1. Gupta, V. K. *et al.* A predictive index for health status using species-level gut microbiome profiling. *Nat. Commun.* **11**, 4635 (2020).
2. Gunathilake, M. *et al.* Alterations in Gastric Microbial Communities Are Associated with Risk of Gastric Cancer in a Korean Population: A Case-Control Study. *Cancers* **12**, 2619 (2020).
3. Huang, Z., L.*et al.* Long-term chronic food-derived arsenic exposure induce the urinary system metabolic dysfunction in mice. Sci Total Environ. 2023