



## BASIC SCIENCE ARTICLE

# Maternal antibiotic exposure disrupts microbiota and exacerbates hyperoxia-induced lung injury in neonatal mice

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**BACKGROUND:** Perinatal antibiotic treatment alters intestinal microbiota and augments hyperoxia-induced lung injury in mice offspring. The effect of maternal antibiotic treatment (MAT) during pregnancy on the lung microbiota and its relationship with lung injury remains unknown.

**METHODS:** We fed timed-pregnant C57BL/6N mice sterile drinking water containing antibiotics from gestational day 15 to delivery. Neonatal mice were reared in either room air (RA) or hyperoxia (85% O<sub>2</sub>) from postnatal days 1 to 7. Four study groups were obtained: control + RA, control + O<sub>2</sub>, MAT + RA, and MAT + O<sub>2</sub>. On postnatal day 7, lung and intestinal microbiota were sampled from the left lung and lower gastrointestinal tract. The right lung was harvested for histology and cytokine analysis.

**RESULTS:** MAT during pregnancy significantly reduced the total number of commensal bacteria in the intestine and birth body weight of newborn mice compared with control newborn mice. Neonatal hyperoxia exposure impaired alveolarization and angiogenesis, which was exacerbated by MAT. Neonatal hyperoxia altered the composition and diversity of intestinal and lung microbiota and MAT further exacerbated neonatal hyperoxia-induced intestinal and lung dysbiosis.

**CONCLUSIONS:** MAT during pregnancy exacerbates hyperoxia-induced lung injury probably through the modulation of intestinal and lung microbiota in neonatal mice.

*Pediatric Research* (2021) 90:776–783; <https://doi.org/10.1038/s41390-020-01335-z>

**IMPACT:**

- MAT during pregnancy reduced the total number of commensal bacteria in the intestine.
- Neonatal hyperoxia altered the composition and diversity of intestinal and lung microbiota.
- MAT exacerbated neonatal hyperoxia-induced intestinal and lung dysbiosis.
- Neonatal hyperoxia exposure impaired alveolarization and angiogenesis, which was exacerbated by MAT.
- Avoiding and carefully using antibiotics during pregnancy is a potential therapeutic target for preventing lung injury in hyperoxia-exposed infants.

**INTRODUCTION**

Premature birth is the leading cause of death in children <5 years old, with the global prevalence range of 5%–18%.<sup>1,2</sup> Improvements in neonatal respiratory care have increased the survival rate of infants with very low birth weight.<sup>3</sup> Supplemental oxygen is often required to treat preterm newborns with respiratory disorders. However, prolonged exposure to high concentrations of oxygen leads to inflammation and acute lung injury.<sup>4</sup> Neonatal rodents reared in hyperoxia exhibited decreased alveolar and capillary development, which is similar to human bronchopulmonary dysplasia (BPD).<sup>5</sup> The term mouse model is suitable for studying preterm birth because preterm infants who develop BPD are delivered in the sacular stage of lung development.<sup>6</sup> Despite early surfactant therapy, optimal ventilation strategies, and increased use of noninvasive positive pressure ventilation, BPD remains a complication of premature births.<sup>7</sup> Therapeutic strategies for hyperoxia-induced lung injury have been in focus

in pediatric medicine, but effective therapies have not been discovered.

The microbiota has been implicated in the regulation of inflammatory, infectious, and metabolic diseases and in causing, preventing, and maintaining the human disease.<sup>8,9</sup> Evidence suggests that the influence of host–microbe interactions extend beyond the local environment to the peripheral tissues.<sup>10</sup> Hyperoxia exposure for the first 2 weeks of life was reported to alter lung microbial diversity in neonatal mice.<sup>11</sup> Maternal antibiotic treatment (MAT) during pregnancy alters gut microbiota and augments hyperoxia-induced lung injury in the offspring.<sup>12–14</sup> However, the effects of MAT during pregnancy on lung microbiota and the relationship between neonatal hyperoxia, the lung and intestinal microbiome, and lung injury remain unknown. The aims of this study are to evaluate the effects of MAT during pregnancy on intestinal and lung microbiota and lung development in hyperoxia-exposed neonatal mice.

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Received: 10 August 2020 Revised: 27 November 2020 Accepted: 2 December 2020

Published online: 19 January 2021

## MATERIALS AND METHODS

### Animals and experimental protocol

Our study was approved by the Animal Care and Use Committee at Taipei Medical University (LAC-2019-0290). Time-dated pregnant C57BL/6N mice were housed in individual cages with a 12-h light–dark cycle and free access to laboratory food and water. The mice were allowed to deliver vaginally at term. In the MAT model, we fed timed-pregnant C57BL/6N mice sterile drinking water containing antibiotics (ampicillin, gentamicin, and vancomycin; 1 mg/mL) used by pregnant women and human newborns, starting from gestational day 15 to delivery.<sup>15</sup> The control dams were fed sterile drinking water. Antibiotics were discontinued immediately after birth, the litters were pooled and randomly redistributed among newly delivered mothers, and the pups were randomly assigned to receive either room air (RA) or hyperoxia (85% O<sub>2</sub>). Four study groups were created as follows: control + RA, control + O<sub>2</sub>, MAT + RA, and MAT + O<sub>2</sub>. The pups in the O<sub>2</sub>-treated (normobaric hyperoxia) group were reared in an atmosphere containing 85% O<sub>2</sub> from postnatal days 1 to 7. The pups in the RA group were reared in RA from postnatal days 1 to 7. The nursing mothers were rotated between the O<sub>2</sub> treatment and RA control litters every 24 h to avoid oxygen toxicity in the mothers. The oxygen-rich atmosphere was maintained in a transparent 40 × 50 × 60 cm<sup>3</sup> plexiglass chamber that received O<sub>2</sub> continuously at 4 L/min. The oxygen levels were monitored using a ProOx Model 110 monitor (NexBioOxy, Hsinchu, Taiwan).

### Determination of the efficacy of maternal antibiotics

At birth, seven mice from the MAT group and five from the control group were sacrificed, and faeces from their lower intestinal tract were used to determine the total bacterial load, which was quantified using quantitative polymerase chain reaction (qPCR) with universal bacterial primers (forward: 5'-AAACTCAAAGGATTGACG G-3'; reverse: 5'-CTCACRRACGAGCTGA-3').<sup>16</sup> Cycling conditions using the Bio-Rad CFX96 thermal cycler were optimized as follows: 95 °C for 3 min; 40 cycles of 95 °C for 15 s, 61 °C for 15 s, and 72 °C for 10 s; and 85 °C for 5 s followed by fluorescence detection. Standard curves were prepared using the genomic DNA of *Bifidobacterium lactis*. The copy number was determined based on a product size of 136 bp using the following equation: copy number = (ng × number/mol)/(bp × ng/g × g/mol of bp).

### Mouse tissue collection and processing

Animals were euthanized with an overdose of isoflurane. The left lung and 2 cm of the lower gastrointestinal tract from the anus to the colon were harvested on postnatal day 7. We sampled microbiota using a culture-independent approach (community sequencing of the 16S rRNA gene, Illumina MiSeq). The instruments were rinsed with ethanol and flamed before each harvest. Murine lungs were excised, placed in tubes containing 1 mL of sterile water, and homogenized mechanically using the Tissue-Tearor (BioSpec Products, Bartlesville, OK). The tissue homogenizer was cleaned and rinsed in ethanol and water between samples. Water control samples from homogenization exposed to clean instruments were sequenced as procedural controls.

### Lung histology

The lung sections were obtained from the right middle lobe and were dehydrated in alcohol, cleared in xylene, and embedded in paraffin. Five-micrometer tissue sections were stained with hematoxylin and eosin and examined using light microscopy to assess lung and intestinal morphometry. Mean linear intercept (MLI), an indicator of mean alveolar diameter, was assessed in ten nonoverlapping fields.<sup>17</sup> Vascular density was determined through von Willebrand factor (vWF) immunohistochemical staining. Microvessel density was determined by counting the number of vessels positive for vWF staining in at least four random lung fields at ×400 magnification in an unbiased manner.<sup>18</sup>

### Immunohistochemical staining for vWF

Immunohistochemical staining was performed on 5-μm paraffin sections. After the paraffin sections were deparaffinized, heat-induced epitope retrieval was performed by immersing the slides in 0.01 M sodium citrate buffer (pH 6.0). To block endogenous peroxidase activity and nonspecific binding of antibodies, the sections were preincubated for 1 h at room temperature in 0.1 M phosphate-buffered saline containing 10% normal goat serum and 0.3% H<sub>2</sub>O<sub>2</sub>. The sections were incubated with rabbit polyclonal anti-vWF antibody (1:100; Abcam, Cambridge, MA) for 20 h at 4 °C and biotinylated goat anti-rabbit immunoglobulin G (1:200, Jackson ImmunoResearch Laboratories, Inc.) for 1 h at 37 °C. The avidin–biotin complex kit (Vector Laboratories, Inc.) and diaminobenzidine substrate kit (Vector Laboratories, Inc.) were used to visualize the brown reaction product, according to the manufacturer's recommendations.

### Lung cytokine levels

The levels of lung tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were determined using the Bio-Plex multiplex assay system (Bio-Rad, Hercules, CA) and Procarta immunoassay kit (Affymetrix) according to the manufacturer's instructions.

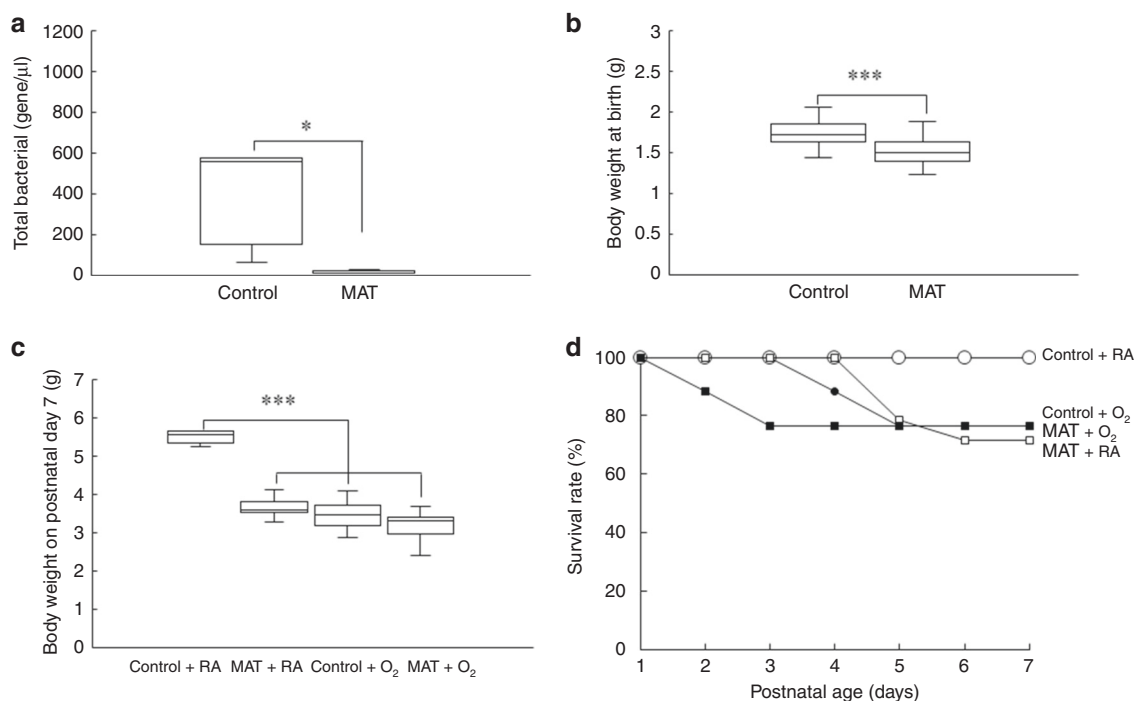
### 16S rDNA gene sequencing and next-generation sequencing analysis

The protocol for 16S rDNA analysis is described by Yang et al.<sup>19</sup> Briefly, 16S rDNA was purified from fecal samples by using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) and from lung tissue by using the QIAamp DNA Microbiome Kit (Qiagen). Library preparation was performed according to the protocol for preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System. Sequence reads have been deposited in the European Nucleotide Archive under the accession number PRJEB28574. The gene-specific sequences used in this protocol target the 16S V3 and V4 region and were removed from the demultiplexed, paired reads using Cutadapt (v1.12). The filtered reads were processed in the R environment (v3.6.1) using R package DADA2 (v1.14.1) following the workflow described by Callahan et al.<sup>20</sup> Taxonomy assignment was performed using the SILVA database (v128) as the reference with a minimum bootstrap confidence of 80. Multiple sequence alignment of the structural variations was performed using DECIPHER (v2.14.0), and a phylogenetic tree was constructed from the alignment using phangorn (v2.5.5).<sup>21,22</sup> The count table, taxonomy assignment results, and phylogenetic tree were consolidated into a phyloseq object, and community analyses were performed using phyloseq (v1.30.0).<sup>23</sup> The alpha-diversity indices were calculated using the estimate\_richness function from the phyloseq package. Statistical comparison between treatment and control was performed with the exact alpha set at 0.05 (Kruskal–Wallis and Wilcoxon's tests). UniFrac distances were calculated using the GUniFrac package (v1.1) to assess community dissimilarity between groups.<sup>24</sup> Principal coordinate analysis (PCoA) ordination on UniFrac distances was performed, and the adonis and betadisper functions from the vegan package (v2.5.6) were used for statistical analysis for the dissimilarity of composition among groups and homogeneity of dispersion, respectively.

Enrichment analysis between groups was performed using the LEfSe (linear discriminant analysis effect size) method with Wilcoxon–Mann–Whitney test ( $\alpha=0.05$ ) and logarithmic linear discriminant analysis (LDA) score >2 and visualized as a cladogram using GraPhlAn.<sup>25,26</sup>

### Statistical analysis

Data are presented as box-and-whisker plots. Statistical analyses were performed using two-way analysis of variance with a Bonferroni post hoc test for multiple group comparisons. The survival rate was evaluated using the Kaplan–Meier method, and a



**Fig. 1 Maternal antibiotic treatment (MAT) reduced total intestinal commensal bacteria and body weight at birth and MAT and postnatal hyperoxia decreased body weight and survival rate.** **a** Total bacteria, **b** body weight at birth, **c** body weight on postnatal day 7, and **d** survival rate in mice born to control or antibiotic-treated dams and exposed to postnatal room air (RA) or hyperoxia. qPCR with universal 16S primers revealed that MAT significantly reduced gut microbial load in newborn mice ( $n = 7$ ) born to antibiotic-treated dams compared with mice ( $n = 5$ ) born to control dams. Mice ( $n = 31$ ) born to antibiotic-treated dams exhibited a significantly lower body weight at birth than mice ( $n = 26$ ) born to control dams. MAT + RA ( $n = 10$ ), control + O<sub>2</sub> ( $n = 13$ ), and MAT + O<sub>2</sub> ( $n = 13$ ) groups exhibited significantly lower body weights on postnatal day 7 than control + RA ( $n = 9$ ) group. The survival rates were comparable among the control + RA, control + O<sub>2</sub>, MAT + O<sub>2</sub>, and MAT + RA groups on postnatal day 7. Data are shown as box-and-whisker plots. \* $P < 0.05$ , \*\*\* $p < 0.001$ , two-way analysis of variance (ANOVA) followed by Bonferroni post test.

log-rank test was used to compare between groups. The alpha-diversity was evaluated using Kruskal–Wallis one-way analysis of variance with a Bonferroni post hoc test. Correlations between Firmicutes/Bacteroidetes ratio and MLI were analyzed using Spearman’s rank correlation test. The differences were considered statistically significant at  $p < 0.05$ .

## RESULTS

Four control dams gave birth to 26 pups; 9 and 17 pups were randomly distributed to the RA and O<sub>2</sub> groups, respectively. Six antibiotic-treated dams gave birth to 31 pups; 14 and 17 pups were randomly distributed to the RA and O<sub>2</sub> groups, respectively.

**Total intestinal commensal bacteria at birth in newborn mice**  
Gut microbiota was reduced in the mice born to antibiotic-treated dams. Results from qPCR universal 16S primers revealed that MAT significantly reduced the number of commensal bacteria in newborn mice compared with control newborns (Fig. 1a).

### Body weight at birth and on postnatal day 7

Mice born to antibiotic-treated dams exhibited a significantly lower body weight at birth than mice born to control dams (Fig. 1b). Mice born to control and antibiotic-treated dams and reared in hyperoxia and mice born to antibiotic-treated dams and reared in RA exhibited significantly lower body weights on postnatal day 7 than mice born to control dams and reared in RA (Fig. 1c). Mice born to antibiotic-treated dams and reared in hyperoxia exhibited a significantly lower body weight on

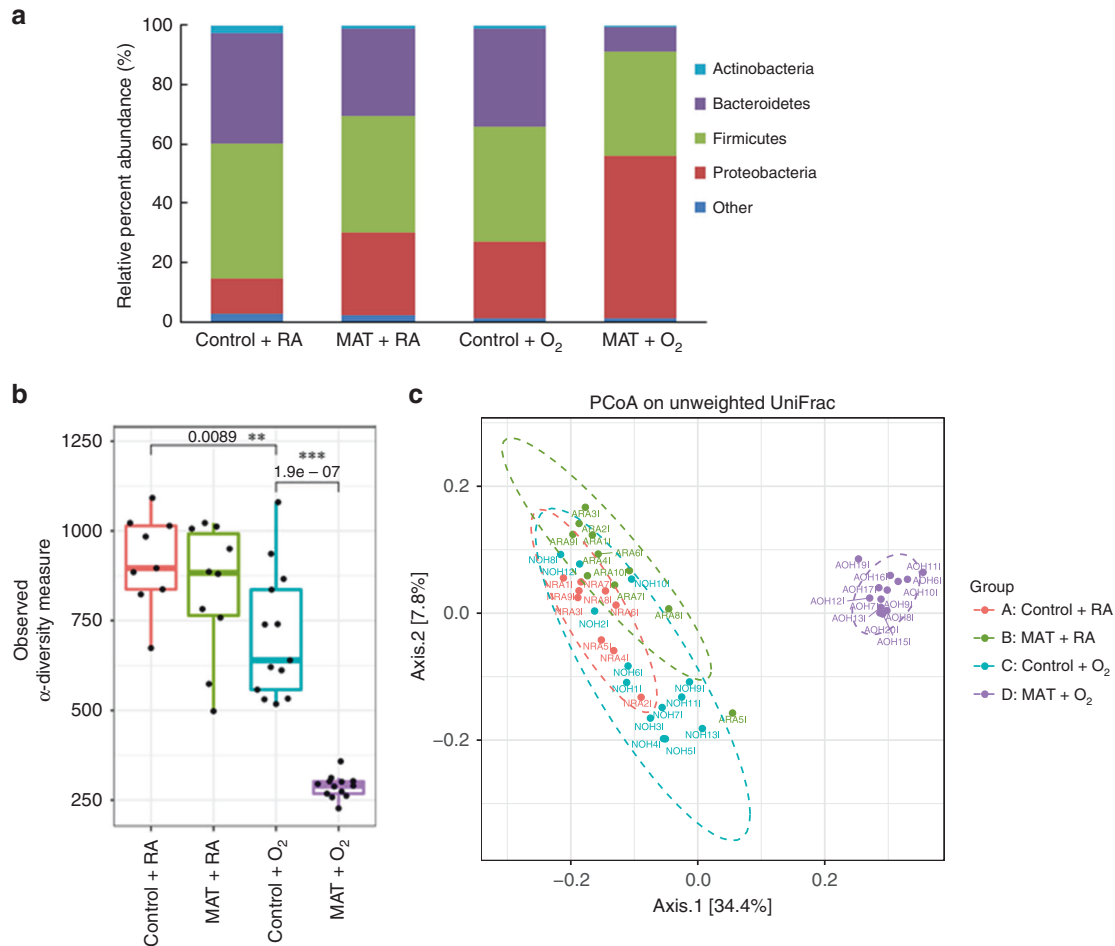
postnatal day 7 than those born to control dams and reared in hyperoxia ( $p = 0.044$ ; Student’s  $t$  test).

### Survival rate

All nine mice born to control dams and reared in RA survived (Fig. 1d). Two mice born to antibiotic-treated dams and reared in hyperoxia died on postnatal days 2 and 3, respectively. Two mice born to control dams and reared in hyperoxia died on postnatal days 4 and 5, respectively. Three mice and one mouse born to antibiotic-treated dams and reared in RA died on postnatal days 5 and 6, respectively. The survival rates were comparable among the control + RA, control + O<sub>2</sub>, MAT + O<sub>2</sub>, and MAT + RA groups on postnatal day 7.

### MAT and postnatal hyperoxia altered the composition and diversity of gut microbiota

We analyzed the taxonomic community structure of the intestinal microbiome on postnatal day 7 to determine its response to hyperoxia (Fig. 2a). At the phylum level, intestinal microbiome in the control + RA, MAT + RA, control + O<sub>2</sub>, and MAT + O<sub>2</sub> groups contained four major bacterial phyla (%): Actinobacteria,  $2.5 \pm 3.1$ ,  $1.3 \pm 1.3$ ,  $0.8 \pm 1.0$ , and  $0.8 \pm 1.5$ , respectively; Bacteroidetes,  $37.3 \pm 8.2$ ,  $29.2 \pm 8.3$ ,  $33.4 \pm 12.0$ , and  $8.2 \pm 12.3$ , respectively; Firmicutes,  $45.9 \pm 4.7$ ,  $39.2 \pm 3.0$ ,  $38.9 \pm 12.4$ , and  $34.9 \pm 8.5$ , respectively; and Proteobacteria,  $11.8 \pm 7.5$ ,  $27.8 \pm 10.8$ ,  $25.6 \pm 19.2$ , and  $54.9 \pm 17.6$ , respectively. The first three phyla accounted for >95% of the sequences in the four groups. MAT and postnatal hyperoxia significantly increased the relative abundance of Proteobacteria and significantly reduced the relative abundance of Bacteroidetes in the intestine compared with the other groups.



**Fig. 2** Maternal antibiotic treatment (MAT) and postnatal hyperoxia altered the composition and diversity of gut microbiota. **a** Bacterial composition at the phylum level, **b**  $\alpha$ -diversity, and **c**  $\beta$ -diversity of intestinal microbiota in 7-day-old mice born to control or antibiotic-treated dams and exposed to postnatal room air (RA) or hyperoxia. Maternal antibiotic treatment and postnatal hyperoxia altered the **a** composition and **b**, **c** diversity of the gut microbiota in 7-day-old offspring. Maternal antibiotic treatment and postnatal hyperoxia increased the relative abundance of Proteobacteria and decreased the relative abundance of Bacteroidetes in the intestine. The gut microbiome from the MAT + O<sub>2</sub> ( $n = 13$ ) group was significantly different from that of the control + RA ( $n = 9$ ), MAT + RA ( $n = 10$ ), and control + O<sub>2</sub> ( $n = 13$ ) groups and exhibited significantly lower  $\alpha$ -diversity than the other three groups. Data are shown as box-and-whisker plots. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

As shown in Fig. 2b, the gut microbiome of the control + O<sub>2</sub> group exhibited significantly lower  $\alpha$ -diversity than the control + RA group. The MAT + O<sub>2</sub> group exhibited significantly lower  $\alpha$ -diversity than the control + O<sub>2</sub> group. The microbiome community structure of each group was assessed using PCoA of unweighted pairwise UniFrac distances. The gut microbiome in the MAT + O<sub>2</sub> group was significantly different from that in the control + RA, MAT + RA, and control + O<sub>2</sub> groups.

#### MAT and postnatal hyperoxia altered the composition and diversity of lung microbiota

We analyzed the taxonomic community structure of the lung microbiome on postnatal day 7 to determine its response to hyperoxia (Fig. 3a). At the phylum level, the lung microbiome in the control + RA, MAT + RA, control + O<sub>2</sub>, and MAT + O<sub>2</sub> groups contained four major bacterial phyla (%): Actinobacteria,  $2.0 \pm 0.7$ ,  $2.2 \pm 1.8$ ,  $0.7 \pm 0.6$ , and  $4.2 \pm 2.4$ , respectively; Bacteroidetes,  $41.6 \pm 0.7$ ,  $45.1 \pm 3.2$ ,  $42.5 \pm 3.8$ , and  $41.5 \pm 4.1$ , respectively; Firmicutes,  $36.7 \pm 2.9$ ,  $33.9 \pm 1.7$ ,  $34.2 \pm 2.9$ , and  $39.0 \pm 3.0$ ; and Proteobacteria,  $17.2 \pm 2.9$ ,  $14.1 \pm 1.2$ ,  $18.2 \pm 4.2$ , and  $10.9 \pm 1.7$ , respectively. MAT and postnatal hyperoxia significantly reduced the relative abundance of Proteobacteria and significantly increased the relative abundance of Actinobacteria in lung microbiota compared with the other groups.

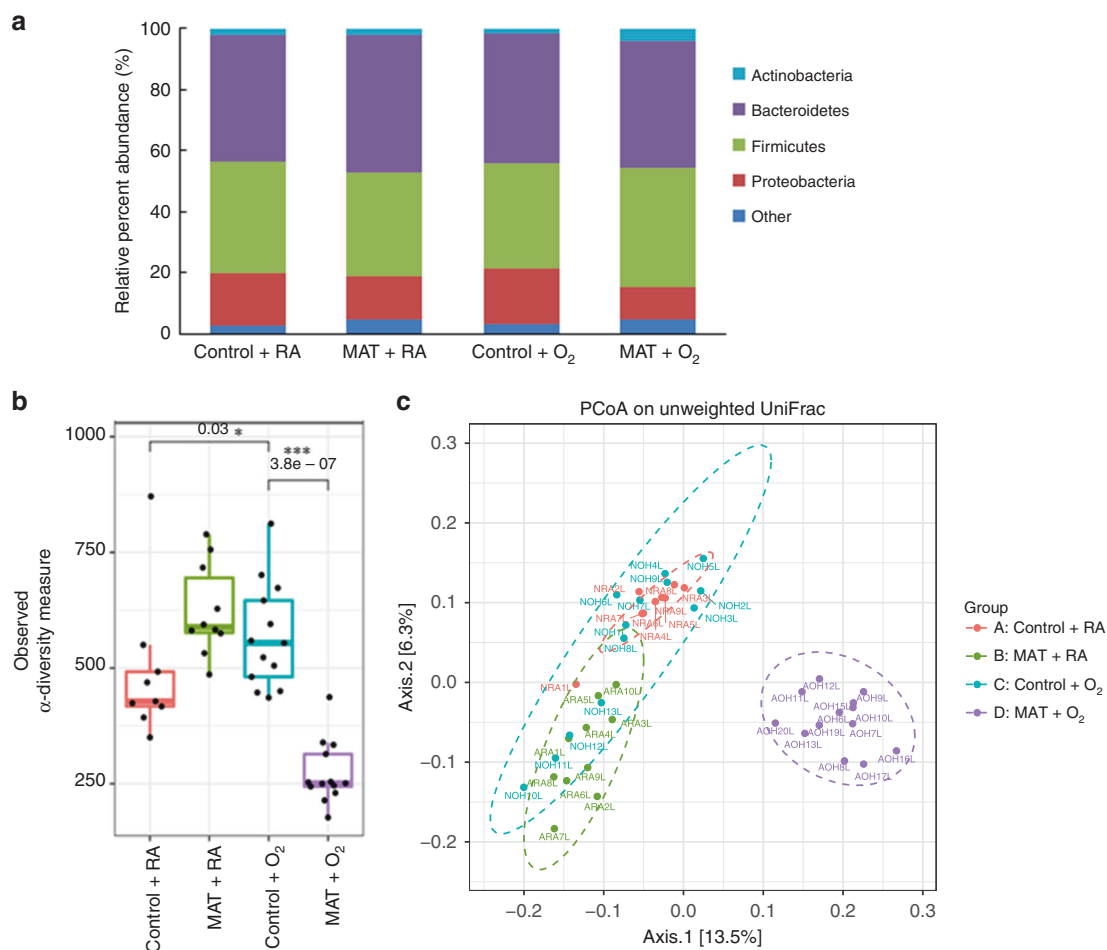
The lung microbiome of the control + O<sub>2</sub> group exhibited significantly higher  $\alpha$ -diversity than the control + RA group. The MAT + O<sub>2</sub> group exhibited significantly lower  $\alpha$ -diversity than the control + O<sub>2</sub> group (Fig. 3b). The microbiome community structure of each group was assessed using PCoA of unweighted pairwise UniFrac distances. The lung microbiome in the MAT + O<sub>2</sub> group was significantly different from that of the control + RA, MAT + RA, and control + O<sub>2</sub> groups.

#### MAT and postnatal hyperoxia altered the intestinal and lung microbiome

To identify microbial taxa affected by MAT and postnatal hyperoxia exposure, LEfSe analysis was performed, which revealed significant differences in relative bacterial abundance at the family level (Fig. 4).

#### Effects of MAT and postnatal hyperoxia on the Firmicutes/Bacteroidetes ratio

The MAT + O<sub>2</sub> group exhibited a significantly higher Firmicutes/Bacteroidetes ratio in the intestinal microbiome than the control + RA, MAT + RA, and control + O<sub>2</sub> groups (Fig. 5a). The Firmicutes/Bacteroidetes ratio in the intestinal microbiome was significantly positively correlated with MLI and significantly negatively correlated with vascular density in neonatal mice lungs (Fig. 5b, c). In the



**Fig. 3 Maternal antibiotic treatment (MAT) and postnatal hyperoxia altered the composition and diversity of lung microbiota.** **a** Bacterial composition at the phylum level, **b**  $\alpha$ -diversity, and **c**  $\beta$ -diversity of the lung microbiota in 7-day-old mice born to control or antibiotic-treated dams and exposed to postnatal room air (RA) or hyperoxia. Maternal antibiotic treatment and postnatal hyperoxia increased the relative abundance of Proteobacteria and reduced the relative abundance of Actinobacteria in the lung. The lung microbiome from the MAT + O<sub>2</sub> ( $n = 13$ ) group was significantly different from that of the control + RA ( $n = 9$ ), MAT + RA ( $n = 10$ ), and control + O<sub>2</sub> ( $n = 13$ ) groups and exhibited significantly lower  $\alpha$ -diversity than the other three groups. \* $p < 0.05$ , \*\*\* $p < 0.001$ , one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

lung microbiome, the MAT + O<sub>2</sub> group exhibited a significantly higher Firmicutes/Bacteroidetes ratio than the MAT + RA and control + O<sub>2</sub> groups; the ratios did not correlate with MLI or vascular density in neonatal mice lungs (data not shown).

#### Lung histology

Representative lung sections stained with hematoxylin and eosin on postnatal day 7 are shown in Fig. 6. The lungs of mice born to control and antibiotic-treated dams and reared in RA exhibited normal lung morphology. The lungs of mice born to control and antibiotic-treated dams and reared in hyperoxia had large thin-walled air spaces and exhibited significantly higher MLI than the lungs of mice born to control or antibiotic-treated dams and reared in RA. The mice born to antibiotic-treated dams and reared in hyperoxia exhibited significantly higher MLI than mice born to control dams and reared in hyperoxia ( $p = 0.028$ ; Student's  $t$  test). Thus, MAT significantly impaired alveolarization in mice reared in hyperoxia.

#### Pulmonary vascular density

Representative lung sections stained for vWF on postnatal day 7 are shown in Fig. 6b. Mice born to control dams and reared in hyperoxia had reduced angiogenesis and exhibited significantly lower vascular density than mice born to control or antibiotic-treated dams and reared in RA. MAT further significantly reduced

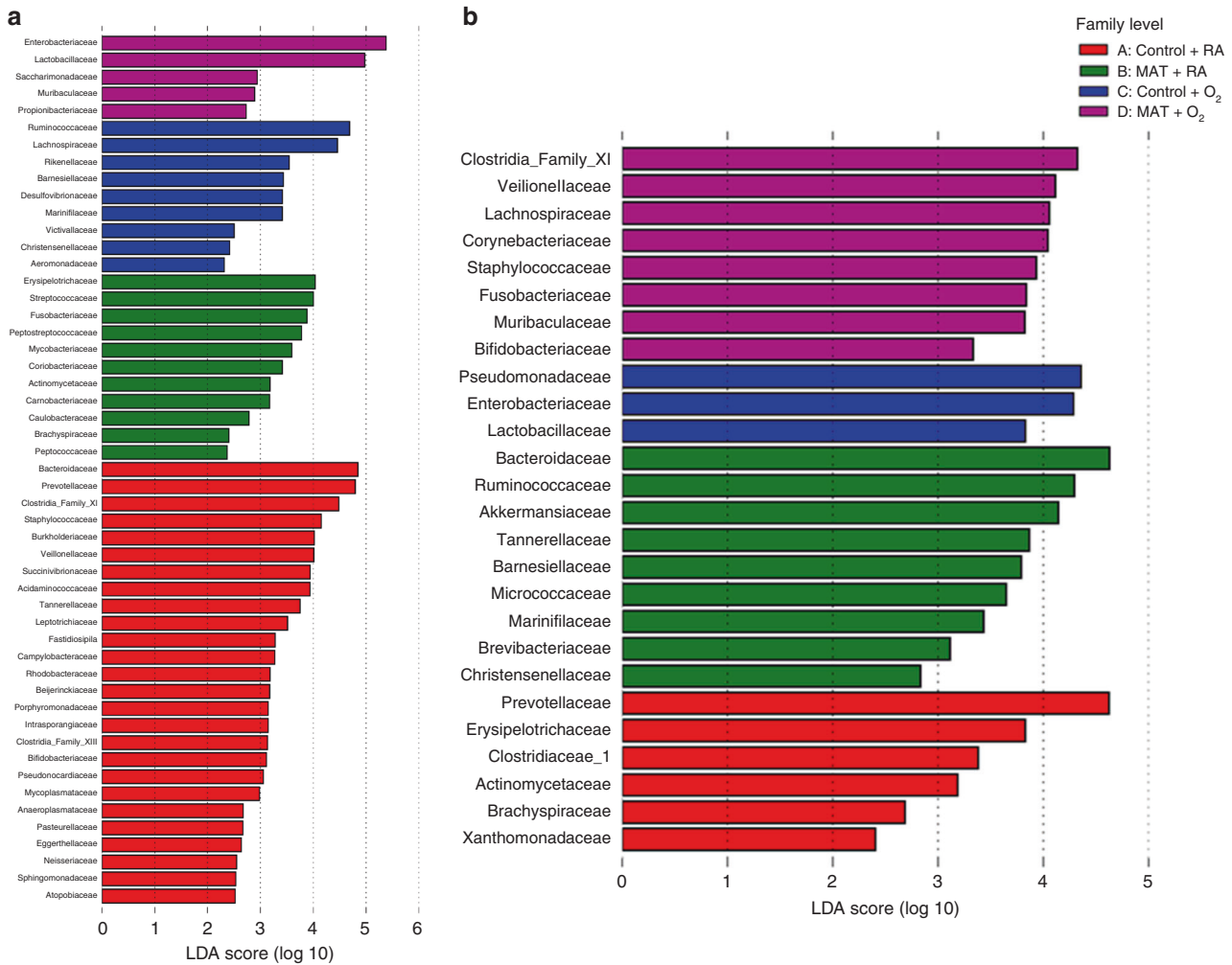
the hyperoxia-induced decrease in vascular density in hyperoxia-reared mice.

#### Cytokine levels in the lung

Control + O<sub>2</sub> group exhibited significantly higher TNF- $\alpha$  and IL-1 $\beta$  levels than did the control + RA and MAT + RA groups (Fig. 6c). MAT + O<sub>2</sub> exhibited significantly higher TNF- $\alpha$  and IL-1 $\beta$  levels than did the control + O<sub>2</sub> group.

### DISCUSSION

Our *in vivo* model demonstrated that MAT during pregnancy suppressed gut microbiota and decreased body weight of the offspring at birth. Neonatal hyperoxia exposure-induced lung injury in mice, as evidenced by increased lung inflammation and reduced alveolarization and angiogenesis. MAT exacerbated neonatal hyperoxia-induced impairment of alveolarization and angiogenesis. Neonatal hyperoxia altered the composition and diversity of intestinal and lung microbiota and MAT exacerbated neonatal hyperoxia-induced intestinal and lung dysbiosis. These findings indicate that MAT during pregnancy exacerbates hyperoxia-induced lung injury through the modulation of intestinal and lung microbiota in neonatal mice. MAT alone did not induce lung inflammation or impair alveolarization and



**Fig. 4 Maternal antibiotic treatment (MAT) and postnatal hyperoxia altered the intestinal and lung microbiome.** Histogram of the linear discriminant analysis scores of bacterial taxa with the family level in the **a** intestine and **b** lung of 7-day-old mice born to control or antibiotic-treated dams and exposed to postnatal RA or hyperoxia. Bacterial taxa significantly differed across the control + RA ( $n = 9$ ), control + O<sub>2</sub> ( $n = 13$ ), MAT + RA ( $n = 10$ ), and MAT + O<sub>2</sub> ( $n = 13$ ) groups identified by LEfSe using the default parameters. RA room air.

angiogenesis in neonatal mice reared in RA. These results suggest that microbiome plays an important role in lung inflammation and development.

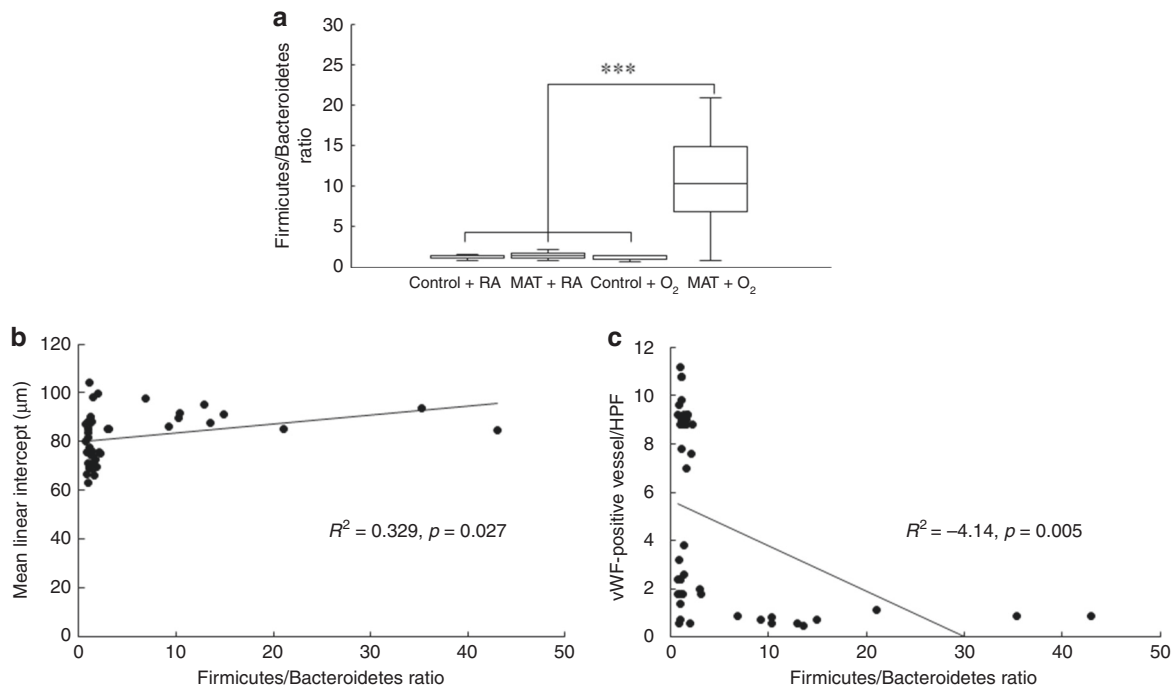
We found that mice born to antibiotic-treated dams exhibited significantly lower body weight at birth than mice born to control dams. On postnatal day 7, mice born to antibiotic-treated dams exhibited significantly lower body weights than those born to control dams and reared in RA. We did not change the number of pups nurtured by the control or antibiotic-treated dams throughout the experiments. MAT tended to significantly reduce body weight in mice reared in hyperoxia. These findings suggest that antibiotic treatment of dams during pregnancy led to a reduced body weight of offspring at birth and had detrimental effects on body weight gain in hyperoxia-reared mice on postnatal day 7. Our findings are compatible with those of Tochtani et al.<sup>27</sup>

We demonstrated that MAT or postnatal hyperoxia exposure increased mice mortality; MAT + postnatal hyperoxia exposure accelerated mice death, although the survival rates were comparable between the control + O<sub>2</sub>, MAT + O<sub>2</sub>, and MAT + RA groups on postnatal day 7. The survival rate did not significantly decrease after MAT or postnatal hyperoxia exposure. These results suggest no association between survival rate and lung development in neonatal hyperoxia-induced lung injury.

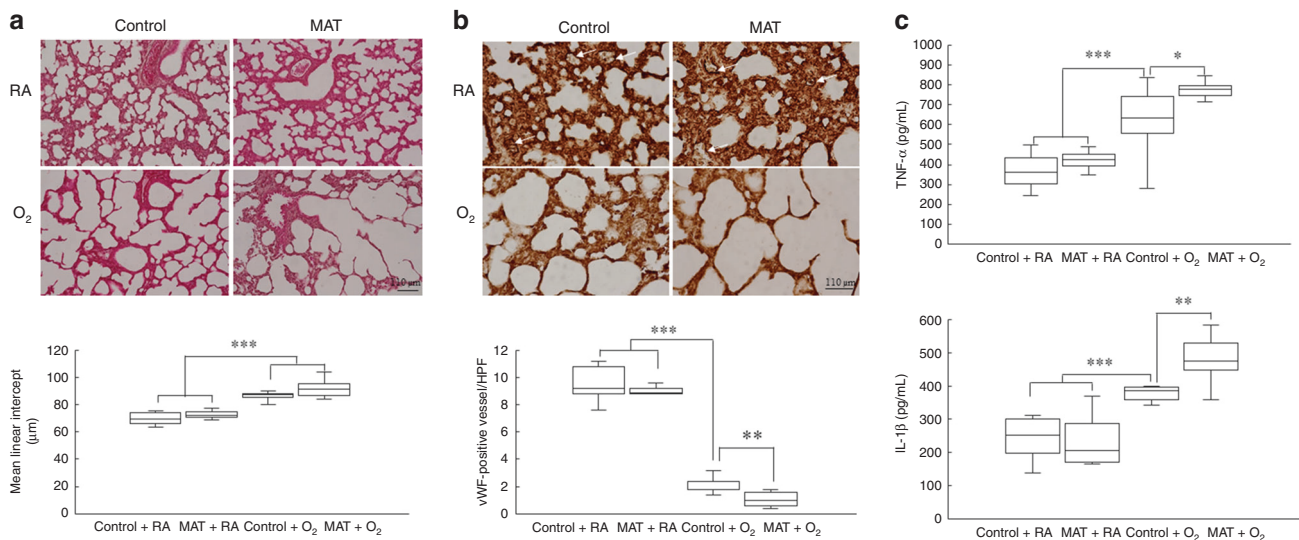
Neonatal hyperoxia exposure has been reported to arrest lung development and perinatal MAT augments neonatal hyperoxia-induced lung injury in mice offspring.<sup>14,28</sup> However, Althouse et al.<sup>29</sup> found that perinatal antibiotic exposure did not worsen lung injury in mice offspring. This discrepancy could be due to differences in the length of MAT and neonatal oxygen exposure. We exposed mice to prenatal antibiotics alone and found that MAT exacerbates hyperoxia-induced lung injury in neonatal mice. These results suggest that further studies are needed to determine the type and duration of maternal antibiotic exposure on hyperoxia-induced lung injury.

Perinatal antibiotic exposure induces intestinal dysbiosis in mice offspring.<sup>14,29</sup> However, the effects of MAT during pregnancy on lung microbiota are unknown. In this study, we demonstrated that neonatal hyperoxia altered the composition and diversity of intestinal and lung microbiota, and MAT exacerbated hyperoxia-induced intestinal and lung dysbiosis. Thus, intestinal and lung dysbiosis was associated with augmentation of hyperoxia-induced lung injury. These findings suggest that antibiotic treatment during pregnancy can alter the inherited microbiome and increase susceptibility to hyperoxia-induced lung injury in the offspring.

The relationship between Firmicutes/Bacteroidetes ratio and obesity was confirmed in humans.<sup>30</sup> The effects of hyperoxia on the Firmicutes/Bacteroidetes ratio of the intestinal and lung



**Fig. 5 Maternal antibiotic treatment (MAT) and postnatal hyperoxia effects on the Firmicutes/Bacteroidetes ratio.** **a** Firmicutes/Bacteroidetes ratio in the intestinal microbiota and correlations between **b** Firmicutes/Bacteroidetes ratio and mean linear intercept and between **c** Firmicutes/Bacteroidetes ratio and vascular density in 7-day-old mice. The MAT + O<sub>2</sub> group exhibited a significantly higher Firmicutes/Bacteroidetes ratio in the intestinal microbiome than the other three groups. The Firmicutes/Bacteroidetes ratio in the intestinal microbiome was significantly positively correlated with mean linear intercept and significantly negatively correlated with vascular density in neonatal mice lungs. RA room air. Data are shown as box-and-whisker plots. \*\*\**p* < 0.001, two-way analysis of variance (ANOVA) followed by Bonferroni post test. Correlations were analyzed using Spearman's rank correlation test. *n* = 9–13 mice.



**Fig. 6 Effects of maternal antibiotic treatment (MAT) and postnatal hyperoxia on alveolarization, angiogenesis, and cytokines in 7-day-old mice.** **a** Representative lung histology and mean linear intercept, control + RA (*n* = 9) and MAT + RA (*n* = 10) groups exhibited normal lung morphology. Control + O<sub>2</sub> (*n* = 13) and MAT + O<sub>2</sub> (*n* = 13) groups contained large thin-walled air spaces and exhibited significantly higher mean linear intercept than control + RA and MAT + RA groups. **b** Representative immunohistochemical staining for von Willebrand factor (vWF) and vascular density and **c** lung cytokines, control + O<sub>2</sub> group exhibited reduced vascularization (arrow) and exhibited significantly lower vascular density and higher cytokines than control + RA and MAT + RA groups. MAT significantly exacerbated hyperoxia-induced decrease in vascular density and hyperoxia-induced increase in cytokines in mice reared in hyperoxia. RA room air. Data are shown as box-and-whisker plots. \**P* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, two-way analysis of variance (ANOVA) followed by Bonferroni post test.

microbiota are unknown. We found that MAT elevated Firmicutes/Bacteroidetes ratios in the intestinal and lung microbiomes, and the ratio in the intestinal microbiome significantly correlated with alveolarization and angiogenesis in neonatal mice. These results

suggest that alterations in the intestinal microbiome contribute to maternal antibiotic-induced exacerbation of hyperoxia-induced lung injury in neonatal mice and manipulation of Firmicutes/Bacteroidetes ratio can be targeted in the prevention of

hyperoxia-induced lung injury. How microbial alterations contribute to the pathophysiology of lung injury remains to be determined. Commensal microbiota may influence lung injury by absorbing micronutrients and generating metabolites that affect lung morphology.

Our study has several limitations. First, we did not examine maternal gut microbiota at the end of antibiotic treatment, although other studies have revealed that the administration of antibiotics to pregnant mice disturbs the maternal and filial gut microbiota.<sup>13,27</sup> Changes in the maternal microbiome may indirectly affect lung development in the offspring through alternate mechanisms. Second, we did not evaluate the bacterial composition of lung microbiota at birth because of the low microbial biomass in mice born to antibiotic-treated dams. Third, we did not measure volumes of water consumed by dams, although a previous study demonstrated that antibiotics administered in drinking water remained relatively stable for 1 week and adult female C57BL/6 mice consumed similar volumes of antibiotic-treated and untreated water.<sup>31</sup>

## CONCLUSIONS

This study demonstrated that neonatal hyperoxia induced intestinal and lung dysbiosis and impaired lung development in mice and that MAT further exacerbated hyperoxia-induced intestinal and lung dysbiosis and lung injury. Currently, no effective therapy for preventing hyperoxia-induced lung injury is available. These findings suggest that maternal antibiotic exposure alters the inherited microbiome and increases susceptibility to hyperoxia-induced lung injury. Avoiding and carefully using antibiotics during pregnancy and manipulating gut microbiota is a potential therapeutic target for preventing and treating lung injury in hyperoxia-exposed infants.

## ACKNOWLEDGEMENTS

This study was supported by a grant from the Ministry of Science and Technology in Taiwan (MOST 109-2314-B-038-073). We would like to acknowledge the technologic and analysis support provided by TMU Core Laboratory of Human Microbiome.

## AUTHOR CONTRIBUTIONS

C.-M.C.: study design, acquisition and analysis of data, drafting, and final approval of the manuscript. Y.-C.S.H.Y.: acquisition and analysis of data, drafting, and final approval of the manuscript. H.-C.C.: acquisition and analysis of data, drafting, and final approval of the manuscript.

## ADDITIONAL INFORMATION

**Competing interests:** The authors declare no competing interests.

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