

Multi-omics reveals the mechanisms of DEHP driven pulmonary toxicity in ovalbumin-sensitized mice

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ABSTRACT

The plasticizer di- (2-ethylhexyl) phthalate (DEHP) is considered a risk factor for allergic diseases and has attracted public attention for its adverse effects on health. However, respiratory adverse effects after DEHP exposure in food allergies have rarely been reported. MiRNAs are considered to be key regulators in the complex interrelationships between the host and microbiome and may be a potential factor involved in DEHP-induced pulmonary toxicity. To investigate the adverse effects of DEHP on the lung during sensitization, we established an ovalbumin (OVA)-sensitized mouse model exposed to DEHP and performed 16S rDNA gene sequencing, miRNA sequencing, and correlation analysis. Our results showed that DEHP aggravated the immune disorder in OVA-sensitized mice, which was mainly characterized by an increase in the proportion of Th2 lymphocytes, and further enhanced OVA-induced airway inflammation without promoting pulmonary fibrosis. Compared with the OVA group, DEHP interfered with the lung microbial community, making *Proteobacteria* the dominant phylum, while *Bacteroidetes* were significantly reduced. Differentially expressed miRNAs were enriched in the PI3K/AKT pathway, which was closely related to immune function and airway inflammation. The expression of miR-146b-5p was elevated in the DEHP group, which was positively correlated with the proportion of Th2 cells and significantly negatively correlated with the abundance of *Bacteroidetes*. The results indicate that DEHP may interfere with the expression of miR-146b-5p, affect the composition of the lung microbiota, induce an imbalance in T cells, and lead to immune disorders and airway inflammation. The current study uses multi-omics to reveal the potential link between the plasticizer DEHP and allergic diseases and provides new insights into the ecotoxicology of environmental exposures to DEHP.

1. Introduction

In recent years, the prevalence and severity of allergic diseases have increased significantly worldwide and have become a major public health problem (Haahntela et al., 2017). Allergic diseases are systemic multiorgan diseases caused by allergen-induced aberrant immune

responses, sometimes involving more than two organs at the same time, such as the gastrointestinal tract and respiratory tract (Vermeulen et al., 2018). It has been reported that approximately 50 % of patients with food allergy have allergic reactions involving respiratory symptoms (James, 2003), but the mechanism remains unclear.

There is growing evidence which supports that exposure to

Abbreviations: DEHP, Di- (2-ethylhexyl) phthalate; OVA, Ovalbumin; Th, T helper; FA, Food Allergy; HE, Hematoxylin-eosin; CVF, Collagen Volume Fraction; ELISA, Enzyme Linked Immunosorbent Assay; IL, Interleukin; FACS, Fluorescence-Activated Cell Sorting; Treg, Regulatory T; QPCR, Quantitative Real-time Polymerase Chain Reaction; DE, Differentially Expressed; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; PCoA, Principal Coordinate Analysis; LEfSe, Linear Discriminant Analysis Effect Size; LDA, Linear Discriminant Analysis; PICRUST, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; ROC, Receiver Operating Characteristic; AUC, Area Under Curve.

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environmental agents with endocrine disrupting properties, such as phthalates (PAEs), may increase the risk of allergic disease development and alter immune function (Benjamin et al., 2017; Bolling et al., 2020; Li et al., 2017). Di- (2-ethylhexyl) phthalate (DEHP), the most common high molecular-weight phthalate, is commonly used as a plasticizer and exists widely and persistently in the environment worldwide. Epidemiological studies have shown that urine levels of DEHP metabolites were statistically higher in children with eczema or wheeze (Bolling et al., 2020; Shi et al., 2018). Animal experiments further showed that DEHP (30 µg/kg.d- 300 mg/kg.d) could act as an immune adjuvant and may lead to the occurrence of allergic diseases by increasing the Th2 immune response (Guo et al., 2012; Wang et al., 2018; You et al., 2016). There is also evidence that oral exposure of DEHP at high concentrations (300 µg/kg.d- 600 mg/kg.d) can cause varying degrees of lung injury (Atia and Abdel-Gawad, 2019; Camacho et al., 2020; Guo et al., 2012). However, the respiratory adverse effects and related mechanisms after DEHP exposure in food allergy have rarely been reported. Therefore, it is of practical significance to explore the pulmonary toxicity and mechanism of DEHP in the process of food allergy.

In addition, studies have shown that complex gene–environment interactions contribute to the rising prevalence of allergic disease (Murrison et al., 2019). As one of the most important epigenetic regulators, miRNA has been proven to be associated with immune and inflammatory disorders in allergic diseases. Exposure to air pollution can lead to changes in lung miRNA expression, which may be an important immunomodulatory mechanism (Weidner et al., 2021). More specifically, indoor air pollution, containing phthalate esters, induces the elevation of serum miR-155 in the asthma group in Chinese children (Liu et al., 2019), which is considered a potential therapeutic target to reduce Th2-mediated inflammation and allergy (Okoye et al., 2014). However data are sparse on how environmental chemicals, such as phthalates, affect the whole miRNA profile in allergic diseases, and the precise mechanisms need to be explored.

The interaction between the microbiome and host plays a key role in regulating the immune system (Geuking et al., 2011). The respiratory microbial communities have been implicated in the pathogenesis of various respiratory diseases, particularly atopic conditions like asthma (Dickson et al., 2016; Marsland et al., 2013). Growing evidence has indicated that the interaction of environmental chemical exposures with the microbiome may lead to altered health status (Jin et al., 2017). A recent study of Latino workers in the United States showed a significant positive correlation between the abundance of *Moraxella* genus in the nasal cavity and DEHP concentration within workers (Dalton et al., 2022), and higher levels of *Moraxella* have been shown to be associated with adverse respiratory health outcomes. However, the relationship between pulmonary toxicity driven by DEHP and the lung microbiota has not been thoroughly studied.

In conclusion, we hypothesized that DEHP may change the microbiota composition and miRNA expression profile in the lungs during the development of food allergy, thereby leading to immune disorder and lung injury. To investigate the mechanisms of pulmonary toxicity of DEHP, we established a mouse model of OVA-induced food allergy and discuss the interactions between immune response, epigenetic and environmental exposure by integrating lung microbiome and miRNA profiling. These results will provide new insights into the ecotoxicology of environmental exposures to DEHP and will also open up the possibility of developing prevention and treatment strategies to reduce the risk of exposure in populations.

2. Materials and methods

2.1. Animals

BALB/c mice (females, 8 weeks old) were housed in specific pathogen-free conditions in the Animal Research Center of the Ruijin Hospital. The mice were fed under a light and dark cycle of 12 h at an

ambient temperature of 23 ± 2 °C. They were randomly divided into three groups (six in each group): the PBS group (PBS), OVA-sensitized group (50 µg OVA/200 µL PBS), and DEHP exposure group (50 µg OVA/200 µL PBS, and 500 mg/kg/d DEHP). These groups were abbreviated as the PBS group (PBS), OVA group (OVA), and DEHP group (DEHP).

2.2. Ethics statement

The animal experimental procedures were approved by the Animal Ethical Committee of the Ruijin Hospital, Shanghai Jiaotong University School of Medicine. Animal studies were conducted according to the guidelines of the National Research Council for the Care and Use of Laboratory Animals [SYXK (Shanghai 2011–0113)].

2.3. Sensitization and challenge

We induced food allergies in mice as described previously (Li et al., 2020b) with some modifications. For sensitization, we intraperitoneally injected sterile PBS or OVA (100 µg, grade V; Sigma—Aldrich, St. Louis, MO, USA) with aluminum potassium sulfate adjuvant (1 mg, Sigma—Aldrich, St. Louis, MO, USA) to mice on Days 0 and 14, respectively. For challenge, the mice in the OVA group were orally administered with 50 µg OVA/200 µL PBS every other day from Days 28–38 (6 times). In the DEHP group, mice were challenged with OVA while being gavaged with DEHP (500 mg/kg/d) from Days 28–38 (11 times). After the final challenge, the mice were sacrificed (Fig. 1A). The DEHP dosage (500 mg/kg/d) we chosen was environmentally relevant. Specifically, daily human exposure to DEHP ranges from 3 to 30 µg/kg/d (Kavlock et al., 2002), but measurements of DEHP in household dust can reach as high as 700 mg/kg (Becker et al., 2004). In addition, certain populations are exposed to high levels of DEHP, such as patients undergoing surgery using DEHP-containing medical devices at doses up to 14 mg/kg/day (FDA et al., 2001).

2.4. Histological analysis

Lungs were excised and inflated with fresh 4 % paraformaldehyde overnight at room temperature. The tissues were then embedded in paraffin and sectioned into five µm sections. Hematoxylin-eosin (H&E) staining was performed to assess morphological changes in the lung and airway epithelium and inflammatory cell infiltration and was observed under bright-field microscopy. The degree of lung inflammation was graded using a semiquantitative scoring system as described previously (Myou et al., 2003). Masson's trichrome staining was performed according to the manufacturer's instructions and was used to determine collagen deposition in the airway. To grade the extent of collagen fiber deposition, ImageJ software was used for semi-quantitative analysis, and the collagen volume fraction (CVF) for each specimen was the ratio of the collagen area of the field to the total area of the field. Pathological scoring was performed by three different individuals, blinded to the experimental methods.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Serum was separated from blood samples obtained after the last challenge. The serum levels of anti-OVA-specific IgE, IgG1, IgG2a, and IgA were measured using ELISA kits according to the manufacturer's instructions. These experiments used the qualitative enzyme immunoassay technique and no obvious cross-reaction or interference existed between the detection index and the analogs.

2.6. Flow cytometry

The composition of CD4 + T-cell subsets in the spleen was detected by flow cytometry. The percentages of Th1, Th2, Th17 and Treg cells in

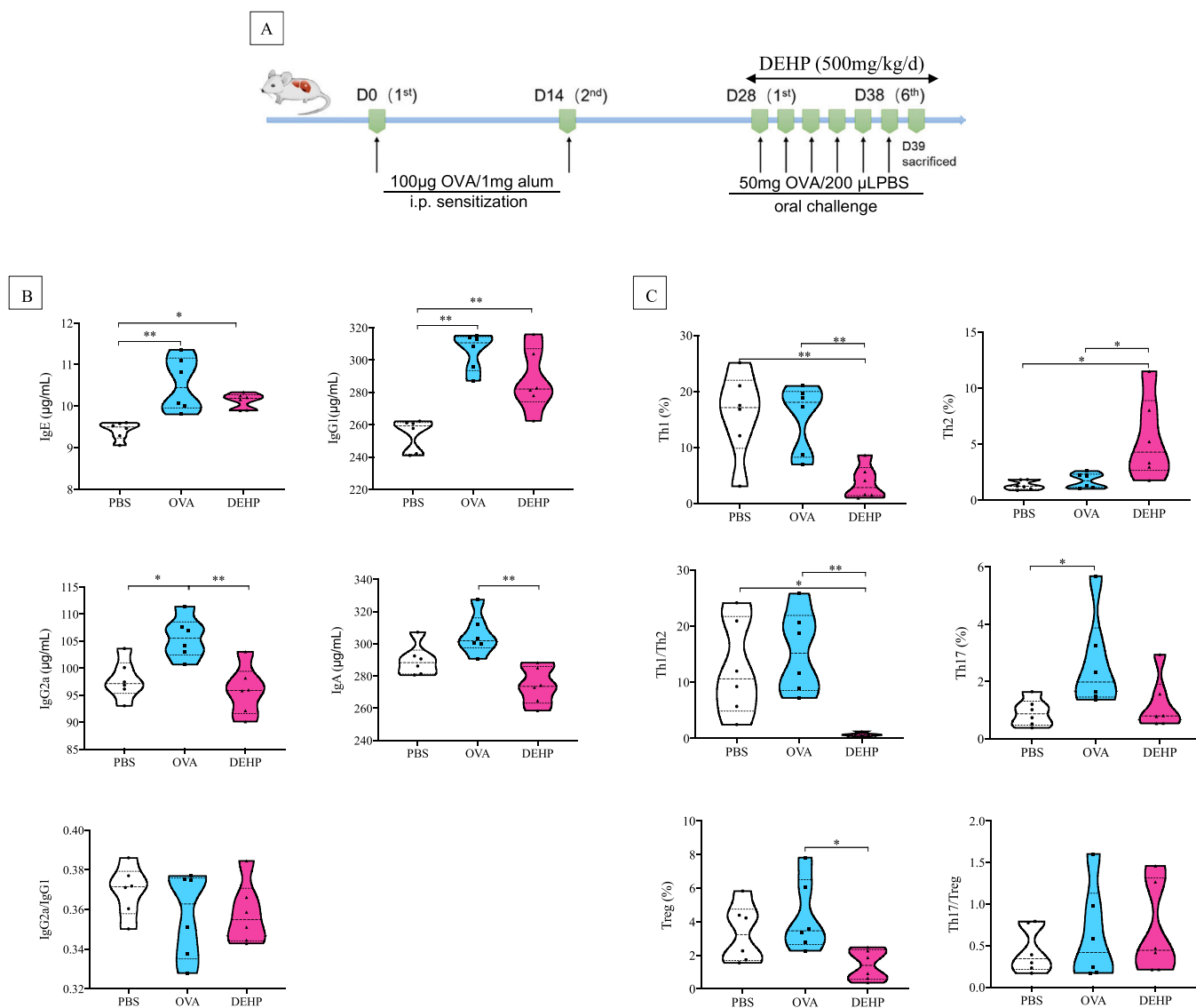


Fig. 1. DEHP aggravates immune disorders in OVA-sensitized mice with high levels of IgE. A. Experimental protocol of sensitization and challenge of the mouse food allergy model. B. The OVA-specific antibody levels in the serum, including OVA-sIgE, OVA-sIgG1, OVA-sIgG2a, OVA-sIgA, and IgG2a/IgG1. C. The proportions of Th1, Th2, Th17 and Treg cell subsets in the spleen. * $P < 0.05$, ** $P < 0.01$.

CD4⁺ lymphocytes were measured. They were defined as CD4⁺IFN- γ ⁺, CD4⁺IL-4⁺, CD4⁺IL-17A⁺, and CD4⁺CD25⁺FoxP3⁺, respectively. The percentage of each subpopulation was determined by its proportion in all CD4⁺ T cells. The data were acquired by flow cytometry on a FACS Celesta system (BD Bioscience, San Diego, CA, USA).

2.7. MiRNA sequencing and data analysis

Total RNA from lung tissue was extracted with TRIzol reagent (Life Technologies, California, USA) according to the manufacturer's protocol. The samples were then delivered to Genesky Biotechnologies Inc. (Shanghai, China) for miRNA sequencing. Raw data were obtained and screened by removing adaptor and low-quality sequences. All the remaining high-quality sequences were blasted with miRBase (<http://www.mirbase.org>) and the Rfam database (<http://rfam.xfam.org/>). To analyze the precursor and mature structures of miRNAs, small RNA sequences were blasted with the corresponding species in the miRBase database by using miRDeep2 software (<https://www.mdc-berlin.de/8551903/en/>). Deseq2 software was used to analyze differentially expressed (DE) miRNAs, and the standards were P values < 0.05 and

$|\log_2(\text{fold change})| > 0.58$. Target genes of differential miRNAs were predicted by the software miRanda and RNAhybrid, and then the intersection of the two was taken as the final result. Enrichment analyses of target mRNAs were analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) in David bioinformatics software.

2.8. Quantitative real-time PCR

To validate the expression of miRNAs, total RNA was extracted from lung tissues using TRIzol Reagent (Takara Biochemicals, China) and then reverse transcribed into cDNA using Reverse Transcriptase M-MLV (RNase H-) (Takara Biochemicals, China) according to the kit's instructions. Quantitative real-time PCR (qPCR) was performed using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Biochemicals, China). The expression of each miRNA was normalized to U6 small nuclear RNA (snRNA) expression. The main PCR protocol was as follows: denaturation for 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. The primer sequences are listed in Table 1. The gene expression levels were quantified using the 2- $\Delta\Delta C_t$ method. In addition,

Table 1
Primer sequences for quantitative real-time PCR.

Gene Name	Primer Sequence (5'–3')
mmu-miR-130b-3p	RT: GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACATGCCC FP: GCGCAGTGAATGATGAAAGG
mmu-miR-146b-5p	RT: GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACAGCCTA FP: GGCGGTGAGAACTGAATTCCATA
mmu-miR-150-5p	RT: GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACCACTGG FP: CCTCTCCCAACCTTGTACC
mmu-miR-133a-3p	RT: GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACCACTG FP: GCTTTGGTCCCTTCAACCA

the forward and reverse primers were designed according to a previous study (Chen et al., 2005).

2.9. Microbiota analysis

DNA from lung samples was extracted with the MagAttract Power-Soil DNA Isolation kit following the manufacturer's guidelines (Qiagen, Hilden, Germany). Amplicon sequencing of the 16 S rRNA gene was carried out to target the V3-V4 variable region by universal primers on the Illumina MiSeq platform (San Diego, CA, USA). The Quantitative Insight into Microbial Ecology (QIIME) was used to process the sequencing data (Caporaso et al., 2010).

The calculation of alpha and beta diversity was performed. The alpha diversity of the bacterial community was represented through some indices including the Chao1, Shannon and Simpson indices. The beta diversity was assessed by principal coordinate analysis (PCoA) of weighted UniFrac distances. The relative abundance of bacterial communities at the phylum level of different groups was determined by the Kruskal–Wallis rank-sum test.

Linear discriminant analysis effect size (LEfSe) analysis was conducted to evaluate the differences in the microbial community and the effect size of each different taxon. The rank for each taxon was assigned based on the corresponding linear discriminant analysis (LDA) score with a threshold (LDA score > 2.0 and $P < 0.05$).

Prediction of microbial function and visualization of gene distribution of functional pathways were performed by phylogenetic investigation of communities by reconstruction of unobserved states 2 (PICRUST2) based on high-quality sequences (Langille et al., 2013) (KEGG <https://www.genome.ad.jp/>).

2.10. Correlation analysis

The correlation between quantitative variables was performed using the Spearman correlation coefficient and the correlation matrices were visualized by a heatmap in R with the pheatmap package. The correlation coefficient (r) values are indicated by a color gradient from blue (negative correlation) to red (positive correlation), as shown in the colored bar.

2.11. Statistical analysis

All values are presented as the mean $\pm$ standard error. GraphPad Prism 8.0 was used for all statistical analyses. Statistical analysis among multiple groups was performed using the ordinary one-way ANOVA. The Mann–Whitney test was used to analyze the differences between two groups. To evaluate the sensitivity and specificity of the parameters to distinguish the DEHP and control groups, the area under the curve (AUC) value for receiver-operating characteristic (ROC) curve was calculated by using SPSS 21.0 software. All statistical tests were bilateral unless otherwise stated. The results with $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. DEHP aggravates immune disorders in OVA-sensitized mice with high levels of IgE

To analyze the sensitization status of the animals, we measured the serum OVA-specific antibodies in our study. The increase in serum IgE, especially allergen-specific IgE, is a typical phenotype of allergic sensitization. IgG1, a subclass of IgG, has regulatory effects similar to those of IgE and has always been considered a surrogate marker for IgE. Therefore, IgE and IgG1 are usually used to evaluate immunogenicity in human or animal models (Pandey, 2013). As shown in Fig. 1B, OVA-IgE and IgG1 levels were significantly increased in both the OVA and DEHP groups compared with the PBS group, while there was no difference between the two groups, suggesting sensitization status of mice. A recent study showed that IgA played a crucial role in allergy prevention in which an impaired IgA response to the gut microbiota was associated with the development of allergic diseases (Dzidic et al., 2017). In our study, the serum OVA-sIgA level was not affected in the OVA group but was decreased in the DEHP group (Fig. 1B). However, the role of IgA in allergies is controversial and needs further study. In addition, DEHP reduced the serum OVA-IgG2a level (Fig. 1B), which predominated in Th1 immune responses.

Lymphocytes isolated from the spleen were stained with surface antibodies specific for CD4 and intranuclear antibodies against IFN- γ , IL-4, IL-17A, and Foxp3 to investigate the influence of DEHP on the percentages of Th1, Th2, Th17 and Treg cells in vivo. As shown in Fig. 1C, there was no significant change in the composition of CD4 + T-cell subsets, except Th17 cells, in the OVA-induced FA mouse model compared with the PBS group. Interestingly, we found that DEHP increased the percentage of Th2 cells and decreased the percentage of Th1 and Treg cells and the Th1/Th2 ratio compared to the OVA group (Fig. 1C).

OVA sensitization led to an allergic state in the mice, but DEHP exposure did not cause the expected aggravation. Since allergies are associated with immune disorders (Tordesillas et al., 2017), we considered whether DEHP can aggravate immune disorders when OVA interferes with immune function. To verify this theory, we performed flow cytometry on the spleen to measure the proportion of T cells. It is generally believed that the imbalance of Th1 and Th2 immune responses and Th2 cell hyperfunction are important pathological bases for allergic diseases, including food allergies and asthma (Nakayama et al., 2017). Previous experimental studies suggested that DEHP can enhance Th2 and Th17 immune responses to exert immunotoxicity (Alfardan et al., 2018), which is partially consistent with our results. Furthermore, FoxP3 + Treg cells can inhibit the inflammatory response mediated by Th2 and Th17 cells and prevent airway inflammation in asthma (Tian et al., 2014). The reduction in Treg cells in the DEHP group (Fig. 1C) may exacerbate the Th2 cell-mediated immune response.

These results together with our findings suggest the following. Mice in both the OVA and DEHP groups were in a sensitized state with high levels of OVA-IgE and IgG1. DEHP exposure did not alter the antibody responses but induced alterations in the proportion of splenic T cells. We suspect that DEHP aggravates immune disorders and that the

immunotoxicity of DEHP may be directly related to antigen-specific Th2 cell hyperfunction and a marked decrease in Th1 cells directly, which is associated with the mechanism of chronic airway inflammation in asthma.

3.2. MiRNAs may be involved in the pathologic changes of airway inflammation aggravated by DEHP

To further elucidate the effects of DEHP immunotoxicity on the lungs in our food allergy model, we conducted a histopathological examination. H&E-stained sections of mouse lung tissue can directly show changes in airway structure and provide intuitive evidence for changes in airway hyperresponsiveness. As shown in Fig. 2 A, the lungs of mice in the PBS group showed a relatively complete alveolar structure without obvious airway inflammation. OVA sensitization slightly increased inflammatory cell infiltration in the alveolar interval and bronchial tissue. Compared with the PBS and OVA groups, the degree of inflammatory cell infiltration, thickening of airway cell layers, and shrinkage of the airway lumen were significantly increased in the DEHP group. Masson's trichrome staining was used to detect thickening of the collagen layer, which is also known as fibrotic hyperplasia in lung tissue. Compared with the control group, the collagen layer was significantly thicker in both the OVA and DEHP groups (Fig. 2 A). Semiquantitative analysis of collagen in lung tissue, expressed as CVF, showed that OVA and DEHP obviously increased collagen fiber deposition ($P < 0.05$), whereas there was no significant difference between them (Fig. 2B). These results showed that in the OVA-induced food allergy model, there

was a slight inflammatory infiltration in the lung tissue and collagen fiber deposition around the small airways. Coexposure to DEHP further enhanced OVA-induced airway inflammation without promoting pulmonary fibrosis and airway remodeling.

MiRNAs are involved in the regulation of immune responses in allergic diseases and are associated with airway inflammation (Pua and Ansel, 2015; Weidner et al., 2021). Therefore, we performed lung miRNA sequencing to further verify the effect of DEHP on the lung. Principal component analysis of samples showed distinct clustering of miRNA profiles of the PBS, OVA and DEHP groups (Fig. 2 C). On the basis of the research criterion $|\log_2 \text{FC}| > 0.585$ and $P \text{ value} < 0.05$, a total of 25 differentially expressed miRNAs were screened after OVA sensitization, among which 7 were upregulated and 18 were downregulated (Table 2). In addition 22 DE miRNAs were screened between the OVA and DEHP groups, among which 6 were upregulated and 16 were downregulated (Table 3). Volcano plots were generated based on the mean expression of the two groups of samples, with a threshold of 0.585 for the fold change in expression (Fig. 2D-E). From the above results, we found that DEHP exposure altered the lung miRNA expression profile.

To confirm the results of miRNA-seq, 4 DE miRNAs were randomly selected. Comparisons of the expression levels of these miRNAs by qPCR are shown in Fig. 2 F. The expression of miR-146b-5p was increased in the DEHP group while the levels of miR-150-5p, miR-133a-3p and miR-130b-3p were downregulated in the OVA and DEHP groups compared to the PBS group, which was similar to the results of miRNA-seq. Notably, miRNA-146b expression was increased in the DEHP group. It has been

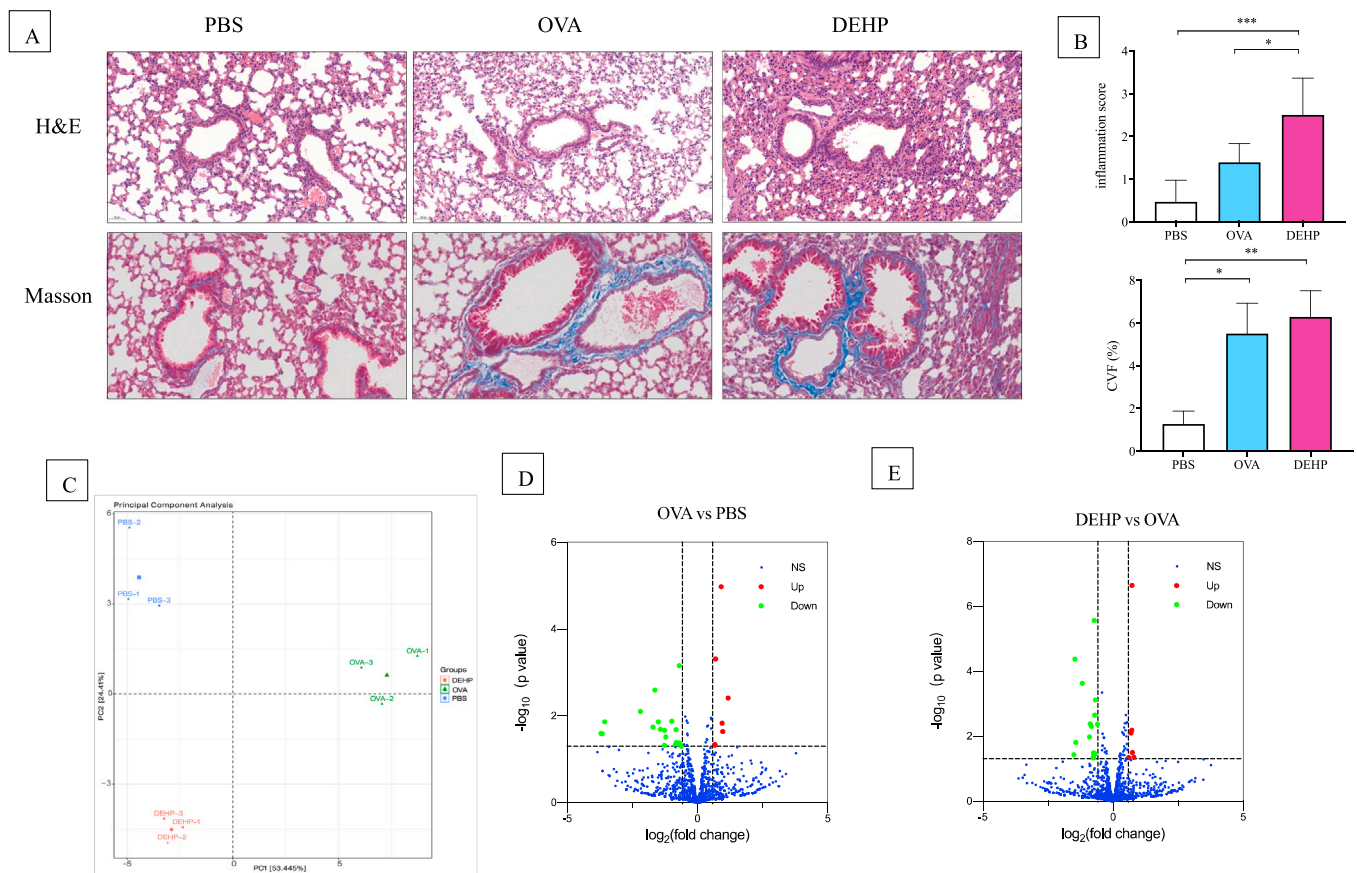


Fig. 2. DEHP aggravates airway inflammation and alters the miRNA profile of lung tissue. A. Histopathology images of the airway structure in lung tissue with HE staining and Masson's trichrome staining (magnification 200 ×). B. The pathological score based on HE staining and Masson's trichrome staining. C. PCA plot showing the separation of miRNAs in the PBS, OVA and DEHP groups. D. Volcano plots of DE miRNAs between the OVA and PBS groups. E. Volcano plots of DE miRNAs between the DEHP and OVA groups. F. Results of qPCR validation: miR-150-5p, miR-133a-3p, miR-146b-5p and miR-130b-3p. G. GO analysis of DE miRNA target genes between the OVA and DEHP groups showing the top 10 significant items from BP, CC and MF. H. KEGG pathways of DE miRNA target genes between the OVA and DEHP groups with $P \text{ value} < 0.05$ and $\text{FDR} < 0.1$ as the cutoff criteria. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

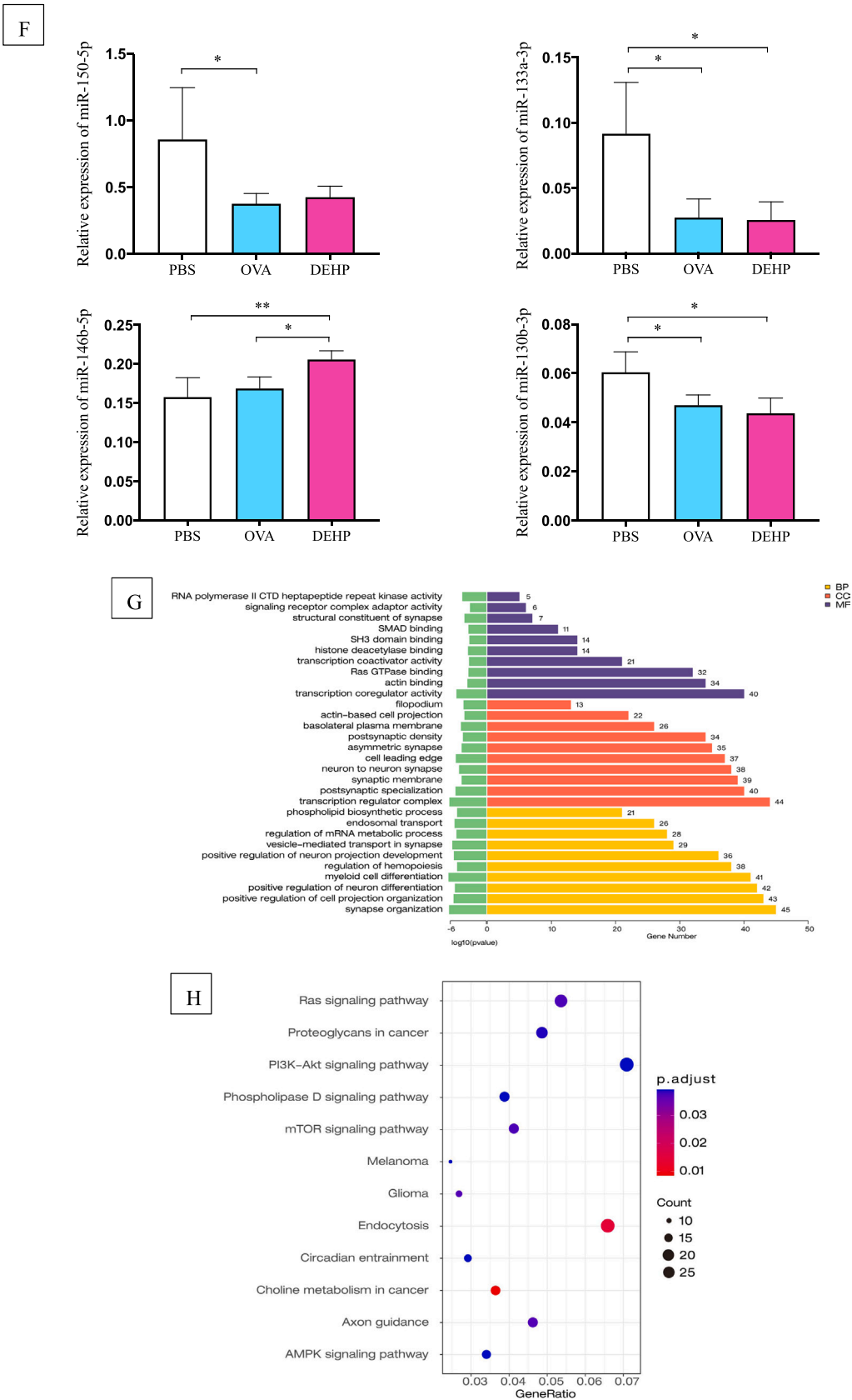


Fig. 2. (continued).

reported that miR-146b was upregulated in the plasma of asthma patients, lung tissue and splenic CD4 T lymphocytes of murine models of asthma (Feng et al., 2012; Garbacki et al., 2011; McGeachie et al., 2017),

which was presumed to be a proinflammatory factor of asthma. These findings may explain the aggravation of airway inflammation after DEHP exposure in our experiments.

Table 2

Differentially expressed miRNA between PBS and OVA groups.

miRNA	PBS	OVA	log2FC	p-value
downregulated				
mmu-miR-9-3p	44.907	13.651	-1.703	0.018
mmu-miR-874-3p	72.904	45.154	-0.693	0.042
mmu-miR-7b-5p	163.758	82.622	-0.978	0.013
mmu-miR-541-5p	347.090	225.873	-0.617	0.050
mmu-miR-495-3p	43.117	15.892	-1.422	0.020
mmu-miR-494-3p	38.957	21.830	-0.813	0.041
mmu-miR-381-3p	2208.935	916.548	-1.269	0.047
mmu-miR-378a-5p	544.874	191.763	-1.505	0.014
mmu-miR-378a-3p	36,795.905	15,844.871	-1.216	0.030
mmu-miR-299a-3p	99.293	54.670	-0.850	0.047
mmu-miR-1a-3p	250,650.734	21,178.084	-3.565	0.014
mmu-miR-150-5p	3872.690	2390.648	-0.696	0.001
mmu-miR-149-5p	1283.059	535.967	-1.259	0.021
mmu-miR-133b-3p	3078.250	236.236	-3.704	0.025
mmu-miR-133a-3p	19,606.044	1560.672	-3.651	0.026
mmu-miR-128-3p	3262.653	1849.327	-0.819	0.021
mmu-miR-10b-5p	8379.089	2686.236	-1.641	0.003
mmu-miR-10b-3p	40.417	8.688	-2.188	0.008
upregulated				
mmu-miR-6412	21.190	41.756	0.974	0.023
mmu-miR-467d-3p	41.632	66.512	0.673	0.047
mmu-miR-467a-3p	41.632	66.512	0.673	0.047
mmu-miR-21c	31.465	70.771	1.176	0.004
mmu-miR-21b	49.208	94.447	0.950	0.015
mmu-miR-15b-3p	260.589	422.254	0.697	0.000
mmu-miR-144-3p	4372.564	8224.665	0.911	0.000

Table 3

Differentially expressed miRNA between OVA and DEHP groups.

miRNA	OVA	DEHP	log2FC	p-value
downregulated				
mmu-miR-3473e	19.820	7.085	-1.503	0.037
mmu-miR-6239	224.359	81.873	-1.461	0.000
mmu-miR-296-5p	24.047	8.840	-1.433	0.015
mmu-miR-6238	120.404	53.314	-1.180	0.000
mmu-miR-1195	71.500	37.962	-0.901	0.011
mmu-miR-6538	110.371	60.028	-0.879	0.004
mmu-miR-3968	200.297	112.271	-0.826	0.005
mmu-miR-130b-3p	44.729	26.435	-0.755	0.043
mmu-miR-2137	53.207	31.334	-0.754	0.047
mmu-miR-467a-3p	70.930	41.791	-0.747	0.033
mmu-miR-467d-3p	70.930	41.791	-0.747	0.033
mmu-miR-144-3p	8777.309	5319.887	-0.722	0.000
mmu-miR-690	891.705	546.266	-0.707	0.002
mmu-miR-193a-3p	61.590	38.380	-0.688	0.035
mmu-miR-136-5p	239.499	149.670	-0.675	0.001
mmu-miR-708-3p	224.657	147.898	-0.597	0.004
upregulated				
mmu-miR-874-3p	48.293	73.733	0.605	0.045
mmu-miR-7b-5p	88.439	143.496	0.690	0.008
mmu-miR-92b-5p	103.497	170.555	0.717	0.007
mmu-miR-146b-5p	47,426.275	78,668.156	0.730	0.000
mmu-miR-448-3p	41.226	68.853	0.747	0.032
mmu-miR-494-3p	23.366	41.101	0.801	0.045

To further understand the potential functions of DE miRNAs, KEGG pathway and GO classification analyses were performed after predicting their target genes (Fig. 2G-H). *P* value < 0.05 and FDR < 0.1 were set as the cutoff criteria. In the GO analysis between groups OVA and DEHP, we list the top 10 significant items from BP, CC and MF in Fig. 2 G. Interestingly, in BP, 3 processes, synapse organization, myeloid cell differentiation and regulation of hemopoiesis may be correlated with immunity. We constructed a KEGG pathway dot plot to show the 12 significantly enriched pathways between OVA and DEHP (Fig. 2H), including endocytosis, the Ras signaling pathway, proteoglycans in cancer, axon guidance, the mTOR signaling pathway, the phospholipase D signaling pathway, choline metabolism in cancer, circadian entrainment, glioma, melanoma, the AMPK signaling pathway and the PI3K-

Akt signaling pathway. Numerous studies have demonstrated that the PI3K/AKT pathway is involved in the regulation of allergic asthma (Athari, 2019), affecting the proliferation of airway smooth muscle and the inflammation of allergic airways (Jiang et al., 2017; Pan et al., 2020). In addition, the administration of PI3K inhibitors can significantly reduce airway inflammation and airway hyperresponsiveness in a murine asthma model induced by sensitization and challenge with OVA (Duan et al., 2005; Kwak et al., 2003). The mTOR pathway has been implicated in immune functions and can regulate the differentiation and activation of CD4 + T-cell subsets (Zheng et al., 2007). mTOR has been reported to be activated during asthma onset, and inhibition of the mTOR pathway in asthmatic mice attenuates asthma markers and reverses Th1/Th2 and Th17/Treg imbalances (Zhang et al., 2017).

The above results showed that DEHP exposure aggravated airway inflammation and altered the lung miRNA expression profile. qPCR further verified the results of miRNA-seq, and showed that the expression of miR-146b, which may be a proinflammatory factor in asthma, was increased in the DEHP group. In addition, the identified molecular pathways in our functional enrichment analysis support the involvement of differential miRNAs in mouse lung pathological changes.

3.3. Lung microbial dysbiosis induced by DEHP may contribute to the exacerbation of airway inflammation

To further elucidate the mechanisms of DEHP immunotoxicity and aggravation of airway inflammation, we investigated the lung microbiome.

An analysis of the mouse lung microbiota diversity was performed as shown in Fig. 3A-B. The alpha diversity, including the Chao1 index, Shannon and Simpson indexes, revealed no differences between the PBS and OVA groups (Fig. 3 A). The microbial richness index (Chao1 index) was significantly higher in the DEHP group than in the other two groups. Of note, the DEHP group showed a slightly lower Shannon index and a markedly higher Simpson index than the OVA and PBS groups, indicating a low microbial community diversity caused by DEHP (Fig. 3 A). The beta diversity shown by principal coordinate analysis (PCoA) based on the weighted UniFrac distances revealed that there were significant differences in bacterial communities among the three groups (ANOSIM $R = 0.307$, $P = 0.0002$) (Fig. 3B).

Fig. 3C shows the bacterial composition and the relative abundance of the three groups, where the bar plot shows that the dominant phyla in the lung microbiota were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* (relative abundance > 0.5 %). In the OVA sensitized group, the composition of lung microbiota differed from that of the PBS group at the phylum level, which was characterized by a higher proportion of *Proteobacteria*, and a lower proportion of *Firmicutes* (Fig. 3D). These two bacteria did not change after DEHP gavage treatment, while *Bacteroidetes* decreased significantly (Fig. 3D). *Proteobacteria* have been reported to be enriched in the airways of asthmatic patients, while the phyla *Firmicutes* and *Bacteroidetes* are less abundant (Hufnagel et al., 2020). These reports are consistent with our results to some extent. Therefore, we can speculate that OVA sensitized mice by interfering with the composition of *Proteobacteria* and *Firmicutes*, while DEHP aggravated it by reducing *Bacteroidetes*.

Furthermore, we conducted LEfSe analyses and LDA scores to identify the most differentially abundant taxa from phylum to genus in lung microbiota (Fig. 3E-F). The data showed that *Eisenbergiella* (*Firmicutes*) were dominant in the PBS group, and *Bacteroidales* (*Bacteroidetes*) could be used as the typical biomarker of the OVA group, while *Proteobacteria* were dominant after DEHP gavage treatment. In other words, the most obvious contrast before and after DEHP intervention was that the abundance of *Bacteroidales* (*Bacteroidetes*) decreased while that of *Proteobacteria* increased.

Multiple studies have reported altered airway microbial dysbiosis and increased abundance of *Proteobacteria* in asthmatic patients (Hilty et al., 2010; Marri et al., 2013). In addition, *Haemophilus* and *Moraxella*,

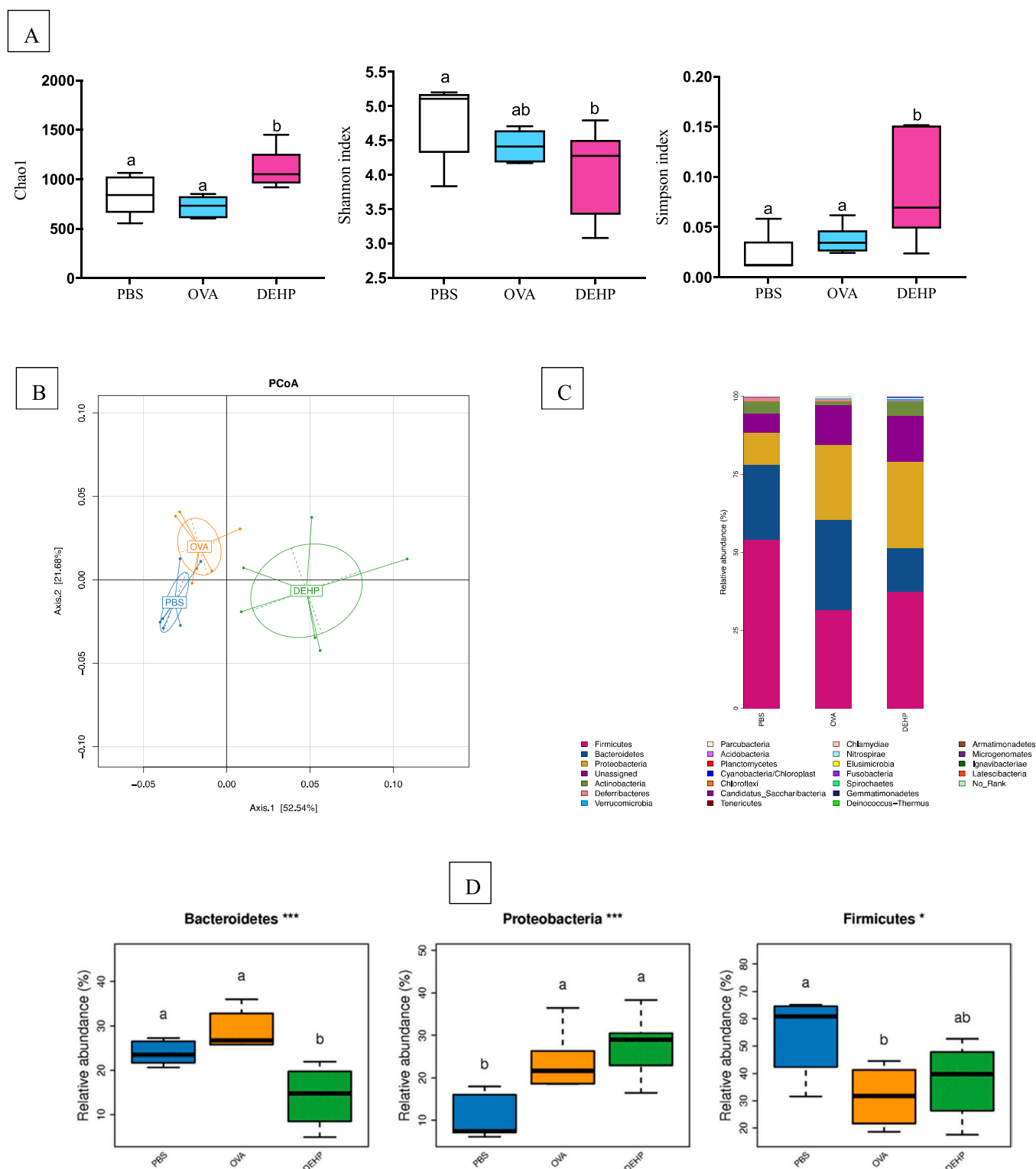


Fig. 3. DEHP alters the composition of lung microbiota. A. The alpha diversity of the lung microbiota, including the Chao1 index, and Shannon and Simpson indexes. B. PCoA of the lung microbiota. PCoA based on the weighted UniFrac distance showed significant differences among the three groups (ANOSIM $R = 0.307$, $P = 0.0002$). C. Relative abundance of lung microbes at the phylum level in the three groups. D. Relative abundance of significantly changed bacterial phyla (*Bacteroidetes*, *Proteobacteria* and *Firmicutes*). E. The cladogram of the lung bacterial community plotted from LEfSe analysis showing the classification levels represented by circle radiates. Different species are colored according to the most abundant group. F. The linear discriminant analysis (LDA) composition of the microbiota. Each bar represents the LDA score of the specific taxon. Higher LDA scores are expressed as longer bars. G. Function prediction of the lung microbiota in the PBS and OVA groups by PICRUST. H. Function prediction of lung microbiota in OVA and DEHP groups by PICRUST. Different letters indicate statistically significant differences in the different groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

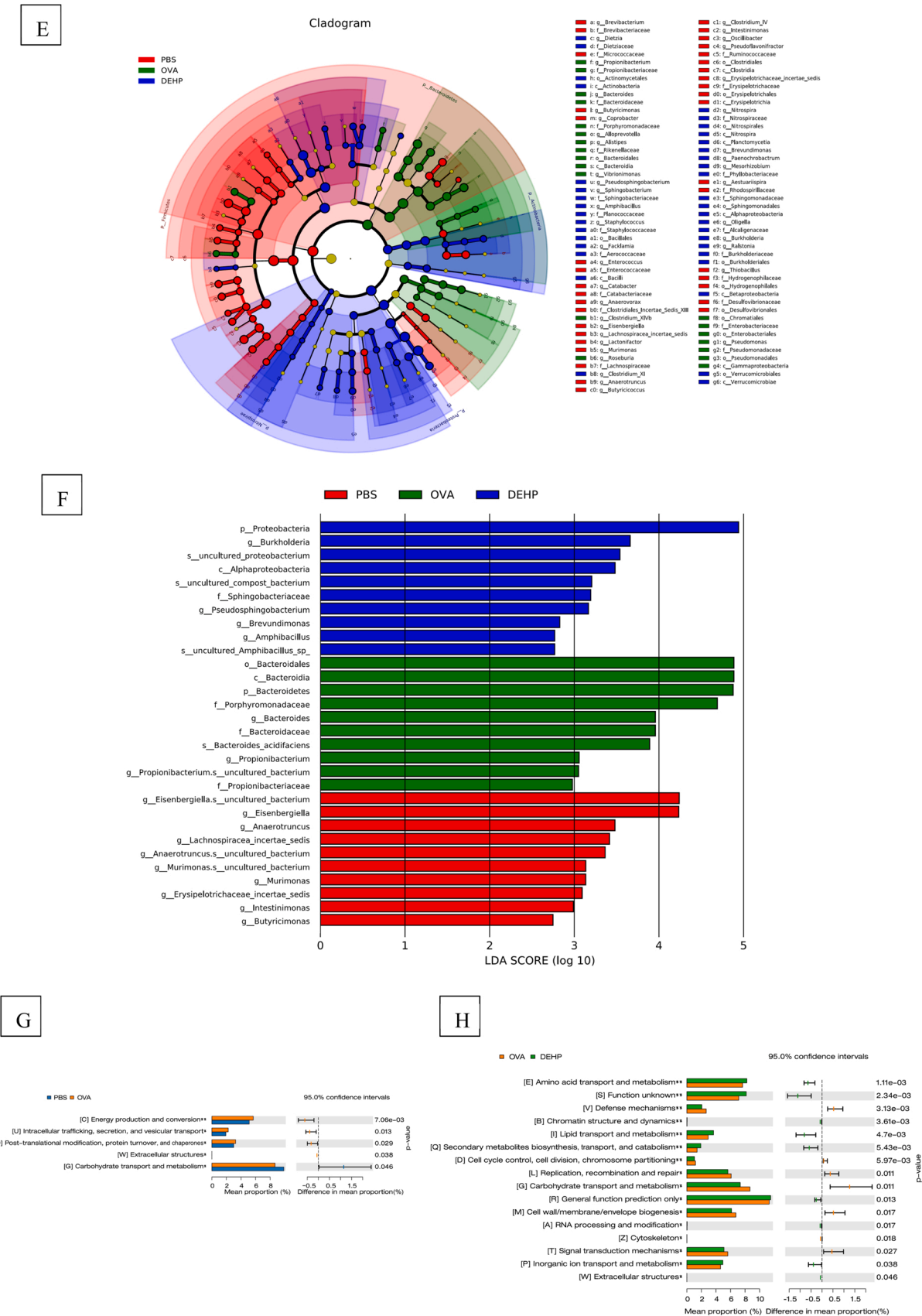


Fig. 3. (continued).

members of the phylum *Proteobacteria*, were found to be significantly increased in asthmatic patients (Hilty et al., 2010). However, Huang and colleagues found that higher abundance of *Proteobacteria* in the airway was associated with asthma exacerbation and bronchial hyper-responsiveness (Huang et al., 2015, 2011). These results support the role of *Proteobacteria* in allergic airway inflammation. In a mouse model in which dietary fiber ameliorated allergic airway inflammation, the proportion of *Bacteroidetes* was increased (Trompette et al., 2014; Zhang et al., 2016), which seems to suggest a protective effect of *Bacteroidetes* on airway inflammation. These findings suggest that DEHP can induce lung microbial dysregulation, including an increase in *Proteobacteria* and a decrease in *Bacteroidetes*, which may lead to the aggravation of airway inflammation, but the specific mechanism is not clear.

According to the changes in the lung microbiota, PICRUST was performed to assess the functional difference in the metabolic pathway caused by OVA and DEHP. As shown in Fig. 3 G in the OVA group, 5 (4 enriched and 1 depleted) functional modules were changed compared with the PBS group. After DEHP exposure, 9 functional modules were enriched (Fig. 3H), which was mainly caused by changes in some metabolism pathways, such as amino acid transport and metabolism; secondary metabolites biosynthesis, transport, and catabolism; lipid transport and metabolism; and inorganic ion transport and metabolism. Studies have now demonstrated that lipids regulate the adaptive and innate immune response, thereby affecting related diseases, including allergies and asthma (Cardoso and Perucha, 2021; Hagemann et al., 2019). Whether these mechanisms are related to DEHP-induced immune disorders remains to be further investigated.

3.4. Correlation analysis reveals the potential relationship between miRNAs, the lung microbiome and T cells

To explore potential links between miRNAs, microbiota and immune function, Spearman's correlation analysis was performed (Fig. 4). Correlation analysis showed that miR-146b-5p was positively correlated with the Th2 cell proportion ($r = 0.66$), and negatively correlated with the Th1-cell proportion ($r = -0.71$) and the Th1/Th2 ratio ($r = -0.81$) (Fig. 4A). The same correlation analysis was also performed on lung bacteria. As shown in Fig. 4B, *Bacteroidetes* exhibited a positive correlation with the Th1-cell proportion and the ratio of Th1/Th2 ($r = 0.64$, $r = 0.71$, respectively), while *Proteobacteria* ($r = -0.83$, $r = -0.59$, respectively) and *Actinobacteria* ($r = -0.72$, $r = -0.69$, respectively) were negatively associated with them. Notably, the Th2 cell proportion was positively correlated with *Proteobacteria* ($r = 0.58$) and *Actinobacteria* ($r = 0.81$) but negatively associated with *Firmicutes* ($r = -0.62$). It has been reported that miRNA-146b can increase the secretion of Th2 cytokines (Specjalski and Jassem, 2019), and is related to the activation and differentiation of T lymphocytes (Baltimore et al., 2008). Some valuable information can be deduced from the above correlation analysis. For example, the increase in Th2 cells, which play an important role in the type 2 immune response, may be the main cause of lung pathological changes, and this immune disorder is closely related to the lung microbiome and miRNA expression. In particular, *Bacteroidetes*, *Proteobacteria* and miRNA-146b may play important regulatory roles. These regulatory networks revealed the immunotoxicity mechanism of DEHP by elucidating the interaction between microbes and host immunity.

In addition, we analyzed the correlation between differential miRNAs and lung microbiota. The results showed that the expression level of miR-146-5p was negatively associated with the abundance of *Bacteroidetes* ($r = -0.83$) (Fig. 4C). We know that the interaction between the

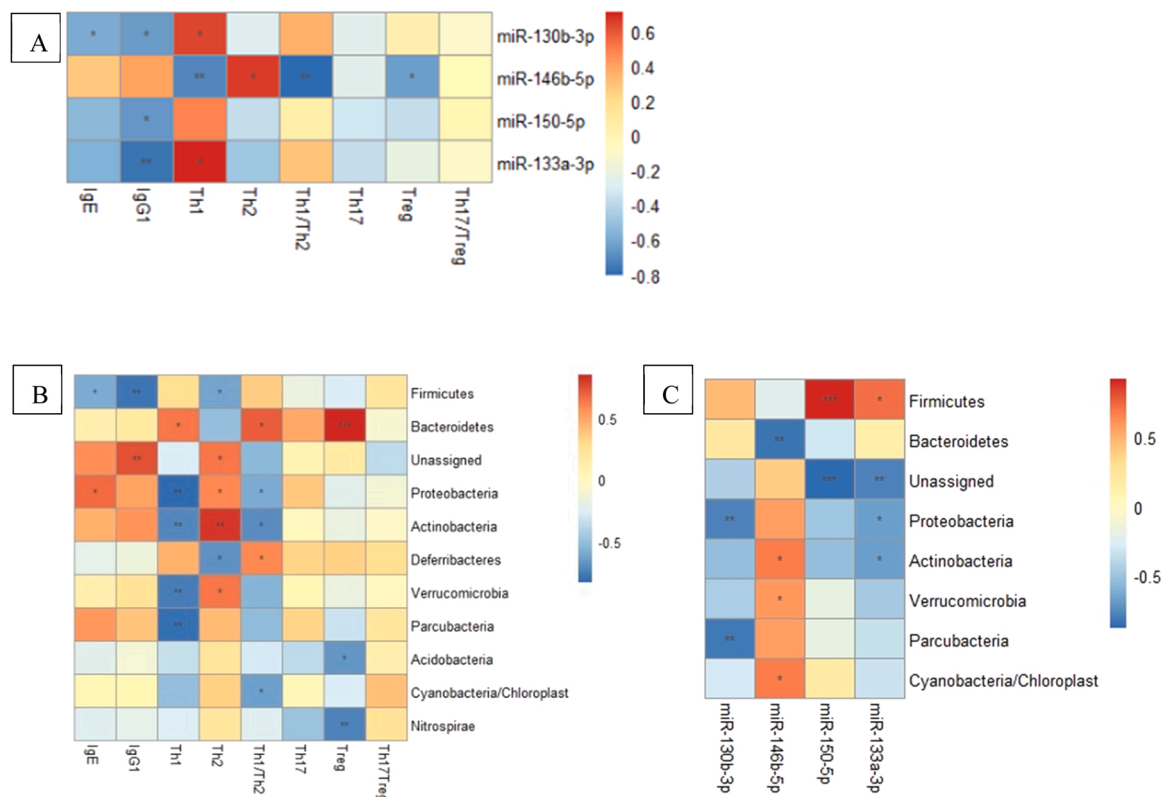


Fig. 4. Correlation analysis of miRNA expression, lung microbiota at the phylum level, immunoglobulins and T cells. Heatmap calculated by Spearman's correlation coefficient for A. miRNA expression and immunoglobulins and T cells. B. Microbiota at the phylum level and immunoglobulins and T cells. C. Microbiota at the phylum level and miRNA expression. The correlation coefficient is indicated by a color gradient from blue (negative correlation) to red (positive correlation), as shown in the colored bar. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

lung microbiota and the host involves complex network regulation. MiRNAs can regulate bacterial growth and affect microbial composition (Liu et al., 2016), while microbiota can modulate host miRNA expression primarily through microbiota metabolites (Li et al., 2020a). Spearman correlation analysis identified a negative correlation between miR-146b-5p and *Bacteroidetes*. This inverse correlation has already been reported. Compared with the controls, the phylum *Bacteroidetes* is less abundant in the airways of adult or child asthmatics (Hilty et al., 2010), while miRNA-146b is upregulated in atopic diseases (including asthma) (Specjalski and Jassem, 2019) and can be used as an asthma exacerbation prediction marker (Kho et al., 2018). In addition, the PICRUST analysis of the lung microbiota showed that the lipid metabolism function was enriched in the DEHP group, while miR-146b was also reported to be involved in the regulation of lipid metabolism during the innate immune response (Ordas et al., 2013).

However, the interaction between the lung microbiota and miRNAs has not been fully discussed, while the significant relationships found in the correlation analysis may suggest that the underlying relationship between lung microbiota and miRNAs may be based on a general host response to dysbiosis involving multiple miRNAs and microbes. Taken together, the above results suggest that there is a close relationship between the airway microbiota and miRNA levels in the immunotoxicity of DEHP.

3.5. Identification of DEHP-related immunodeficiency using ROC curve analysis

The ROC curve was carried out to evaluate the specificity and sensitivity of the above parameters to distinguish DEHP-related immune dysregulation from controls. The AUC was calculated to compare the diagnostic value (Fig. 5). We found a high accuracy for the variables Th1 (AUC=0.917, 95 % CI =0.742–1.000), Th2 (AUC=0.944, 95 %

CI=0.814–1.000) and miR-146b-5p (AUC=0.883, 95 % CI=0.679–1.000), which provided new biomarkers for diagnosis. The AUC values of lung *Proteobacteria* and *Bacteroidetes* were 0.972 and 0.944 respectively, indicating good predictive performances. The AUC of the combined diagnostic model was 1.000, proving the effectiveness of the differential diagnosis. This study highlights the unique immunophenotype of mice exposed to DEHP, which may have profound implications for future targeted therapies in this patient population.

4. Conclusion

Studies have shown that the interaction between the microbiome and the host is crucial in regulating the immune system. Previous studies have found that DEHP plays an auxiliary role in allergic airway diseases by interfering with immune balance (Alfardan et al., 2018), but there have been few studies on its impact on the lung microbiome. Recently, miRNAs have been reported to be key regulators involved in the complex interrelation between the host and the microbiome (Li et al., 2020a).

In this study, we established an OVA-sensitized mouse model and integrated multi-omics analysis of the lung microbiome and miRNA in an attempt to reveal the mechanism of lung injury after DEHP exposure. DEHP aggravated lung inflammation and immune disorders under OVA sensitization, mainly increasing the proportion of Th2 cells in the spleen and reducing the Th1/Th2 ratio. The decrease in *Bacteroidetes* and the increase in *Proteobacteria* were the most significant in the DEHP group, while the lung transcriptome analysis showed significant changes in the expression level of miRNA-146b-5p. Furthermore, the above changes were significantly correlated with the proportion of Th2 cells. Our findings suggest that lung microbiota and miRNA expression are associated with immunity, thereby affecting lung inflammation.

This study uses multi-omics to provide a potential link between

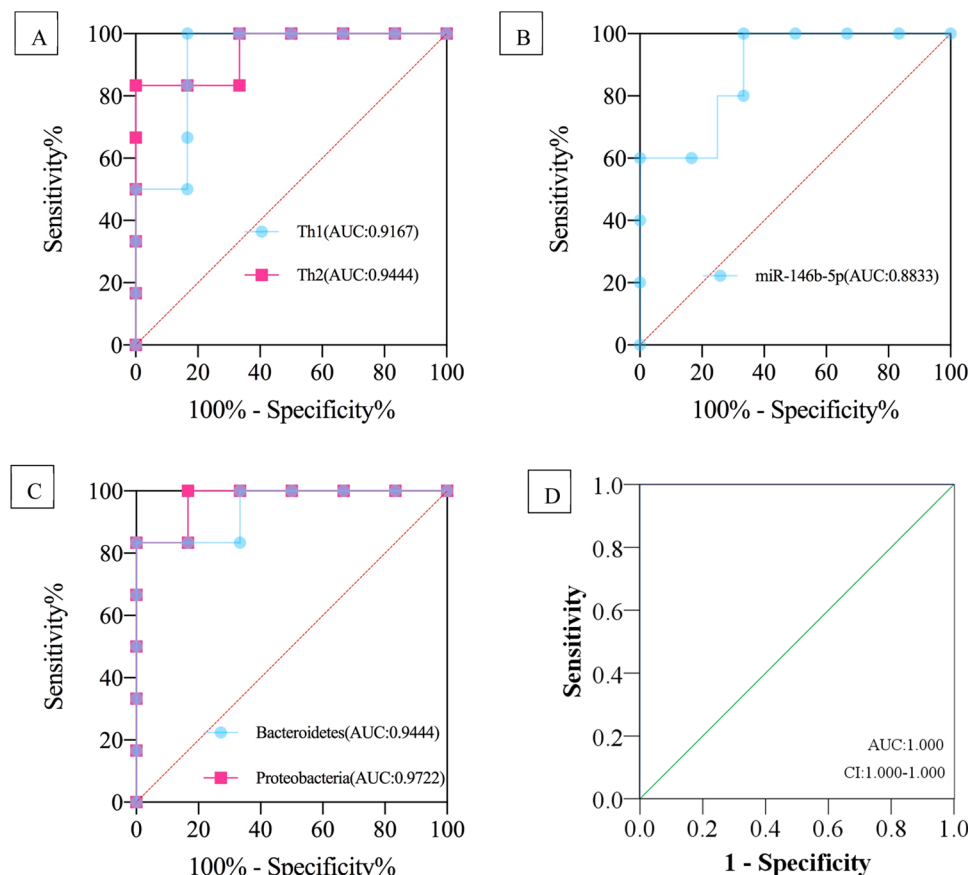


Fig. 5. The receiver operating characteristic (ROC) curve describes the predictive accuracy of the parameters that distinguish the DEHP and control groups. A. ROC (spleen CD4 + T cells). B. ROC (miR-146b-5p). C. ROC (lung microbiota). D. Joint diagnostic ROC. The closer the AUC value is to 1, the better the diagnostic effect. An AUC value between 0.7 and 0.9 indicates certain accuracy, an AUC value between 0.5 and 0.7 indicates low accuracy, and an AUC value under 0.5 indicates no diagnostic value.

environmental plasticizers and immune diseases, providing new insights into DEHP-driven mechanisms of airway inflammation. However, the specific roles of lung microbiota and miRNAs in regulating DEHP-induced immunotoxicity remain unclear, which prompted us to further study them.

CRedit authorship contribution statement

Ping Liu drafted the manuscript. Xu Quan conducted laboratory experiments and carried out the data collection. Qingqing Zhang and Yanyan Chen participated in the data processing. Ping Liu and Xinqiong Wang analyzed the omics data and made the figures. Na Li and Chundi Xu designed the study. The final version of the manuscript was reviewed and approved by all of the authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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