Original Article

Imbalance of intestinal flora activates inflammatory response contributing to acute lung injury

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Background: Seawater drowning is a leading cause of accidental injury and death, and the resulting acute lung injury (ALI) is a serious clinical syndrome for which there are no effective treatments. This study aims to investigate the potential mechanism of seawater drowning-induced ALI.

Methods: Seawater drowning mouse models were constructed to assess lung injury. The hematoxylin & eosin (H&E) staining and fluorescent terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay were used to observe the pathology of lung tissues and apoptosis, respectively. 16S rRNA and RNA-seq were performed to identify the structure of the intestinal microbes and the gene expression profiles of the lung tissue of the mice, respectively. The expression of cytokines was detected by quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), and the activities of superoxide dismutase (SOD) and malondialdehyde (MDA) were detected by assay kits.

Results: The results showed that seawater drowning aggravated lung injury and accelerated cell apoptosis in mice. Seawater exposure significantly altered the structure of mouse intestinal microbes, especially increasing the abundance of *Firmicutes* and decreasing that of *Bacteroidota*. Transcriptional upregulation of inflammatory responses in ALI mice was observed in the lung transcriptome, and differentially expressed genes were mainly enriched in inflammation-related pathways such as cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor, and chemokine signaling pathway, which were further confirmed by microbe-gene association analysis. Furthermore, inflammatory factors were up-regulated, oxidative stress molecule MDA was elevated, and SOD was decreased in the lung tissues of mice, suggesting that the imbalance of intestinal flora activated inflammatory and oxidative stress responses.

Conclusions: This study reveals the mechanism that intestinal microflora aggravates ALI by modulating inflammatory signaling pathways, depicting the landscape of the microbial-gene-lung axis and providing new insights into the use of gut flora as a therapeutic strategy for ALI.

Keywords: Seawater drowning; acute lung injury (ALI); microbial-gene-lung axis; inflammation

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Introduction

Drowning is a critical public safety issue and is the third leading cause of accidental death, occurring especially in children and youth (1). Drowning claims the lives of 372,000 people worldwide each year, and the incidence of non-lethal drowning is much higher than death, according to the statistics of the World Health Organization (2). The severity of the consequences of drowning depends on the composition and amount of water inhaled. Acute lung injury (ALI) induced by seawater drowning is a serious clinical syndrome characterized by an increase in pulmonary alveolar-capillary permeability, an excessive inflammatory response, and refractory hypoxemia (3,4). Both saline and freshwater can destroy lung surface-active substances, and increase the permeability of alveolar-capillary membranes, allowing inflammatory cells to infiltrate into the alveolar space, releasing pro-inflammatory cytokines, reactive oxygen species (ROS), and other molecules, which can lead to further exacerbation of pulmonary edema and impeded gas exchange (5,6). The severity of seawater-induced pulmonary edema is three times higher compared to freshwater-induced edema (7). Although pathophysiology and molecular mechanisms have been investigated, little studies are available on the key microbial and gene expression profiles and the mechanisms underlying seawater-induced ALI.

Seawater drowning is a complex multicascade biological process, and the intestinal microbes have been described as the most important micro-ecosystem in symbiosis with the human body. The lungs were thought to be sterile in the past, but research has now demonstrated that the microbiome does exist in the lungs and that they are altered in a wide range of respiratory diseases (8,9). Investigations have shown that alterations in the intestinal microbiota have a profound effect on the lung function, with the two working in close crosstalk and performing essential functions related to nutrient metabolism, immunity, and biotransformation of bacterial metabolites, a communication known as the gut-lung axis (10,11). This axis is a bidirectional communication pathway between the gut and the lungs that involves a variety of mechanisms, including the exchange of microbial metabolites, interactions of the immune system, and modulation of the inflammatory response (12). Increasing evidence suggest that disturbances in intestinal flora homeostasis are strongly associated with ALI, and one study found that antibiotic cocktail-induced microbiome depletion prevented lipopolysaccharide (LPS)-induced ALI via the gut-microbiota-lung axis (13,14). Metabolites produced by the gut flora, such as short-chain fatty acids (SCFAs), reach the lungs through the circulation and have an impact on lung inflammation and immune responses (15,16). In ALI caused by intestinal ischemia-reperfusion (I/R), succinic acid accumulated in the lungs during intestinal I/R originates from the gut flora, and succinic acid promotes alveolar macrophage polarization and apoptosis of alveolar epithelial cells through its receptor SUCNR1, which leading to lung injury (17). The relationship between intestinal flora and lung disease is complex and clinically relevant, providing new strategies for the prevention and treatment of lung disease.

To explore the host-microbe associations in drowning-induced ALI, we constructed seawater-drowned mouse models to directly mimic the direct effects of seawater drowning on the lungs, in contrast to previous studies that may have used chemical induction or other indirect methods to mimic ALI. The molecular mechanisms of lung injury were analyzed in depth at the microbiome and transcriptome levels by pathological staining combined with 16S rRNA and RNA-sequencing (RNA-seq) techniques, revealing that seawater exposure alters the structure and pathways of action of mouse gut microbes. This exploration of microbe-gene-lung axis interactions highlights the potential of gut flora to treat ALI therapy by modulating inflammatory signaling pathways, and that an integrated strategy combining gut microbial modulation, anti-inflammatory therapy, and antioxidant therapy may be more effective than a single therapeutic approach,

Highlight box

Key findings

- This study investigated the severity of acute lung injury (ALI) in a seawater drowning mouse model. The microbial-gene-lung axis of seawater-induced ALI was identified, providing a novel perspective on the role of gut flora in lung health and disease.

What is known and what is new?

- ALI is a severe condition without effective treatment options, and that gut microbiota can influence health outcomes.
- The mechanism by which the gut microbiota exacerbates ALI by modulating inflammatory signaling pathways illustrates the interplay between gut microbiota, gene expression and lung health.

What is the implication, and what should change now?

- Our study highlights that modulation of the gut microbiota may be a potential therapeutic strategy for ALI, especially when seawater drowning is involved.

providing a more comprehensive therapeutic regimen for treating or mitigating ALI by modulating the gut microbial homeostasis. We present this article in accordance with the ARRIVE reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-633/rc).

Methods

Seawater and animals

Seawater with salinity of 35‰ was prepared according to the previous study (18). Thirty male BALB/c mice (6 weeks old, 20–25 g body weight) were purchased from Hunan SJA Laboratory Animal Co., Ltd. Mice were housed in sterilized cages at room temperature (23–25 ℃), humidity (65%±5%), and a 12-hour light-dark cycle, with free access to food and distilled water, and acclimatized for 7 days for experiments. All experiments were performed in accordance with relevant laws and institutional guidelines for the care and use of animals, and approved by the Laboratory Animal Welfare and Ethics Committee of the Army Medical University [No. SYXK (YU) 2022-0018], in compliance with the national or institutional guidelines for the care and use of animals.

Seawater drowning model and animal treatments

Acute lung injury model induced by seawater drowning in mice was established by immersing mice into seawater of 6 cm depth and 25±2 ℃ for 28 seconds, and then removing the mice and performing cardiopulmonary resuscitation immediately. No mice died during the modelling process. Mice were randomly divided into two groups (n=15): control group and seawater drowning group. At the end of the experiment, the mice were weighed, anesthetized and sacrificed by intraperitoneal injection of 7% chloral hydrate, and lung tissue, stool and blood samples were collected for further experiments. A protocol was prepared before the study without registration.

Tissues collection and histological analysis

The left lower lung tissue was collected 7 days after drowning, dried up the surface liquid, and weighed and recorded as wet weight. The lung tissue was baked in an electric thermostatic drying at 60 ℃, and the weight remained unchanged was recorded as the dry weight, and the wet/dry weight (W/D) ratio of the lungs was calculated. The lung tissues were fixed with 4% paraformaldehyde, and the paraffin-embedded sections were stained with hematoxylin & eosin (H&E). The degree of injury was evaluated by observing the histopathological and morphological conditions. Paraffin sections were routinely dewaxed and rehydrated, and apoptosis was detected by using One step TUNEL apoptosis assay kit (C1089, Beyotime, Shanghai, China). The images were collected under a fluorescence microscope, and apoptotic cells were defined as those with red fluorescence in the nucleus.

Detection of cytokines

The whole blood was collected from mice, and serum was obtained at 4 ℃ overnight and centrifuged at 1,000 ×g for 15 min for further evaluation. The levels of TNF-α, IL-1β, IL-6, and IL-10 were determined with an enzyme-linked immunosorbent assay (ELISA) kit. The levels of superoxide dismutase (SOD) and malondialdehyde (MDA) were determined using Total Superoxide Dismutase Assay kit with NBT (S0109, Beyotime, Shanghai, China) and Lipid peroxidation MDA assay kit (S0131S, Beyotime, Shanghai, China), respectively.

16S rRNA gene sequencing

Genomic DNA was extracted from stool samples by Magnetic Soil and Stool DNA Kit (DP712, Tiangen) for high throughput 16S rRNA gene sequencing. The purity and concentration of DNA were determined by 1% agarose gel electrophoresis, and the sample was diluted to 1 ng/μL with sterile water in a centrifuge tube. The V4 region of the bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using bacterial universal primers 515F 5'-GTGCCAGCMGCCGCGGTAA-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3' (19). Library construction was performed using the NEB Next® Ultra™ II FS DNA PCR-free Library Prep Kit (New England Biolabs, MA, USA). The purified amplicons were sequenced on the NovaSeq 6000 system. Raw data were filtered out of barcodes, primers and other low-quality information to obtain high-quality data. Sequences were compared using the SILVA database (https://www.arb-silva.de/). Effective Tags sequences of all samples were clustered into operational taxonomic units (OTUs). Species annotation was performed using the QIIME2 software based on the Silva 138.1 database and relative abundance of each species was counted. Alpha and beta diversity in

the samples were assessed, including Rarefaction Curves, the Chao1, ace, shannon and simpson index, principal component analysis (PCA), principal co-ordinates analysis (PCoA), nonmetric multidimensional scaling (NMDS) and unweighted pair-group method with arithmetic mean (UPGMA). Furthermore, the key biomarkers were assessed in different groups using LEfSe linear discriminant analysis (LDA) effect size to analyze species strengths and differences. Functional annotation prediction of OTUs was performed using Tax4Fun (V0.3.1). Relationships between environmental factors and species abundance were analyzed based on the Spearman correlation index. The original data generated and analyzed can be obtained from NCBI (Accession ID: PRJNA1075655).

RNA sequencing of lung tissue

Total RNA was extracted from lung tissue samples using the TRIzol method. RNA purity, concentration and integrity were assessed by a Nanodrop 2000 spectrophotometer (Thermo Fisher, MA, USA), a Qubit 3.0 fluorometer (Thermo Fisher, USA) and an Agilent 2100 system (Agilent Technologies, USA), respectively. Sequencing libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, MA, USA) according to the manufacturer's recommendations and RNA-seq was performed on the NovaSeq 6000 platform. After filtering out splice sequences, N-containing reads and reads with Qphred ≤20 using fastp, raw data were aligned to the Mus musculus reference genome (GRCm38) by HISAT2 software. FeatureCounts was used to calculate the number of reads mapped to each gene. Fragments per kilobase per million reads (FPKM) was then calculated for each gene based on the length of the gene and the number of reads mapped to the reference. Differentially expressed genes (DEGs) were analysed using the DESeq2 package. False discovery rate (FDR) <0.05 and |log2(fold change)| >1 were set as the thresholds for significant differential expression. Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome and disease ontology (DO) enrichment analyses were performed on DEGs using clusterProfiler. Gene set enrichment analysis (GSEA) was performed for all genes using clusterProfiler for GO, KEGG, DO and Reactome databases. The original data generated and analyzed during the current study can be obtained from NCBI (Accession ID: GSE255726).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from lung tissues using the TRIzol reagent method. cDNA was synthesized using EntiLink™ 1st Strand cDNA Synthesis Super Mix (ELK Biotechnology, Wuhan, China). qRT-PCR was performed using the EnTurbo™ SYBR Green PCR SuperMix kit (ELK Biotechnology, China) on a QuantStudio 6 Flex System PCR (Life Technologies, CA, USA), with 3 replicates per sample. Relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method with GAPDH as an internal control.

Statistical analysis

Experimental data were processed by SPSS 21.0 statistical software (IBM Corp. Armonk, NY, USA), and visualized by GraphPad Prism (v9.00) software (La Jolla, CA, USA). Measurement data were expressed as mean ± standard error of the mean. One-way analysis of variance (ANOVA) or independent *t*-test was used for comparisons between two groups. P<0.05 indicated statistical significance.

Results

Seawater induced aggravation of lung injury in mice

The seawater drowning-induced ALI mice models were constructed, and the morphological indexes of lung tissue were examined after 7 days. Gross morphologic anatomy of lung tissues showed obvious lung hyperemia and edema after seawater exposure, with an increase in lung wet weight and an elevated W/D ratio (*Figure 1A*,*1B*). H&E-stained sections showed disruption of alveolar space, thickening of alveolar wall and inflammatory cell infiltration after seawater drowning (*Figure 1C*). The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis further showed that significant apoptosis occurred in the lungs of seawater drowned mice, which was highly correlated with lung parenchymal injury (*Figure 1D*). These pathologic changes were not observed in control mice. This suggested that pulmonary edema in the lung tissue of mice was significantly aggravated after seawater drowning.

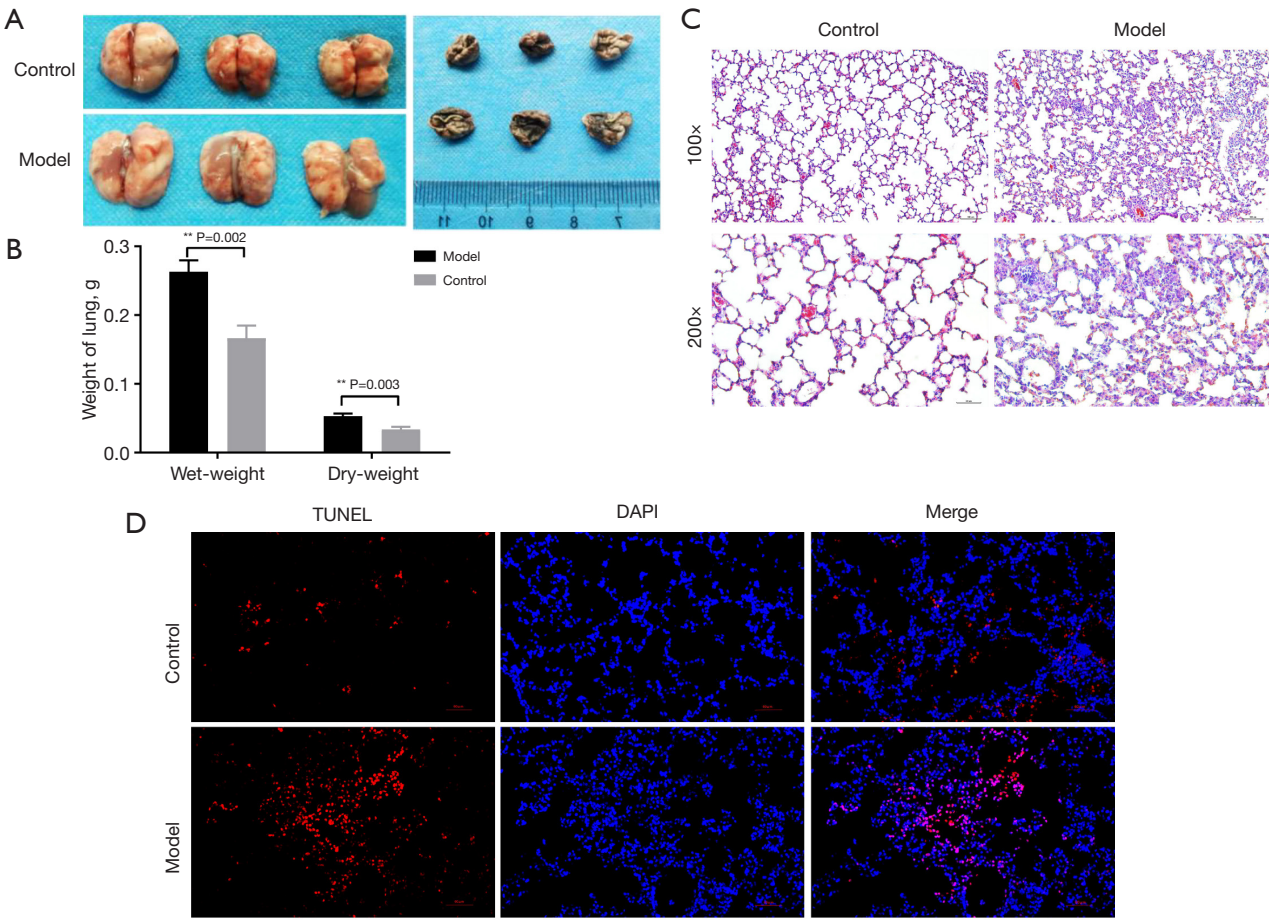

Figure 1 Seawater drowning induces pathological damage in mice. (A) General morphology of mouse lung. (B) Lung wet and dry weights in mice. **, P<0.01. (C) Histopathology of lung tissues from mice examined by hematoxylin & eosin staining. (D) Proportions of apoptotic cells in lung tissues of rats examined by fluorescent TUNEL (200×). Red fluorescence represents apoptotic cells. Model represents mice with acute lung injury induced by seawater drowning.

Effect of seawater drowning on the diversity and abundance of microbes in mice

To investigate whether seawater drowning alters the composition and abundance of the intestinal microbes, 16S rRNA sequencing of stool samples was performed. Compared with the control group, 33.41% of specific sequences were recognized in the ALI mice (*Figure 2A*). In order to identify the dominant microbes, a bar chart of species abundance was drawn based on the results of annotation at various taxonomic levels. As can be seen, there was a significant difference in microbial community composition between the two groups, indicating that seawater drowning modified the intestinal microbes of mice. At the phylum level, *Bacteroidota* and *Firmicutes* were the predominant phylum in two groups (*Figure 2B*). The ratio of *Firmicutes* to *Bacteroidota* was significantly higher in the seawater-drowned group, as evidenced by an increase the abundance of *Firmicutes* and a slight decrease in *Bacteroidota*. There was also an enrichment of *Verrucomicrobiota*, *Deferribacterota* and *Actinobacteriota*. Regarding the genus level, *Muribaculaceae* and *Lactobacillus* were the dominant groups in both groups (*Figure 2C*). *Lachnospiraceae_NK4A136_group*, *Akkermansia*, and *Clostridia_UCG-014* were enriched in seawater drowning-induced ALI group. The results showed that the microbial composition was largely consistent between the two groups, but the proportion of microbial composition differed significantly, demonstrating highly differentiated microecology of the intestinal microbes.

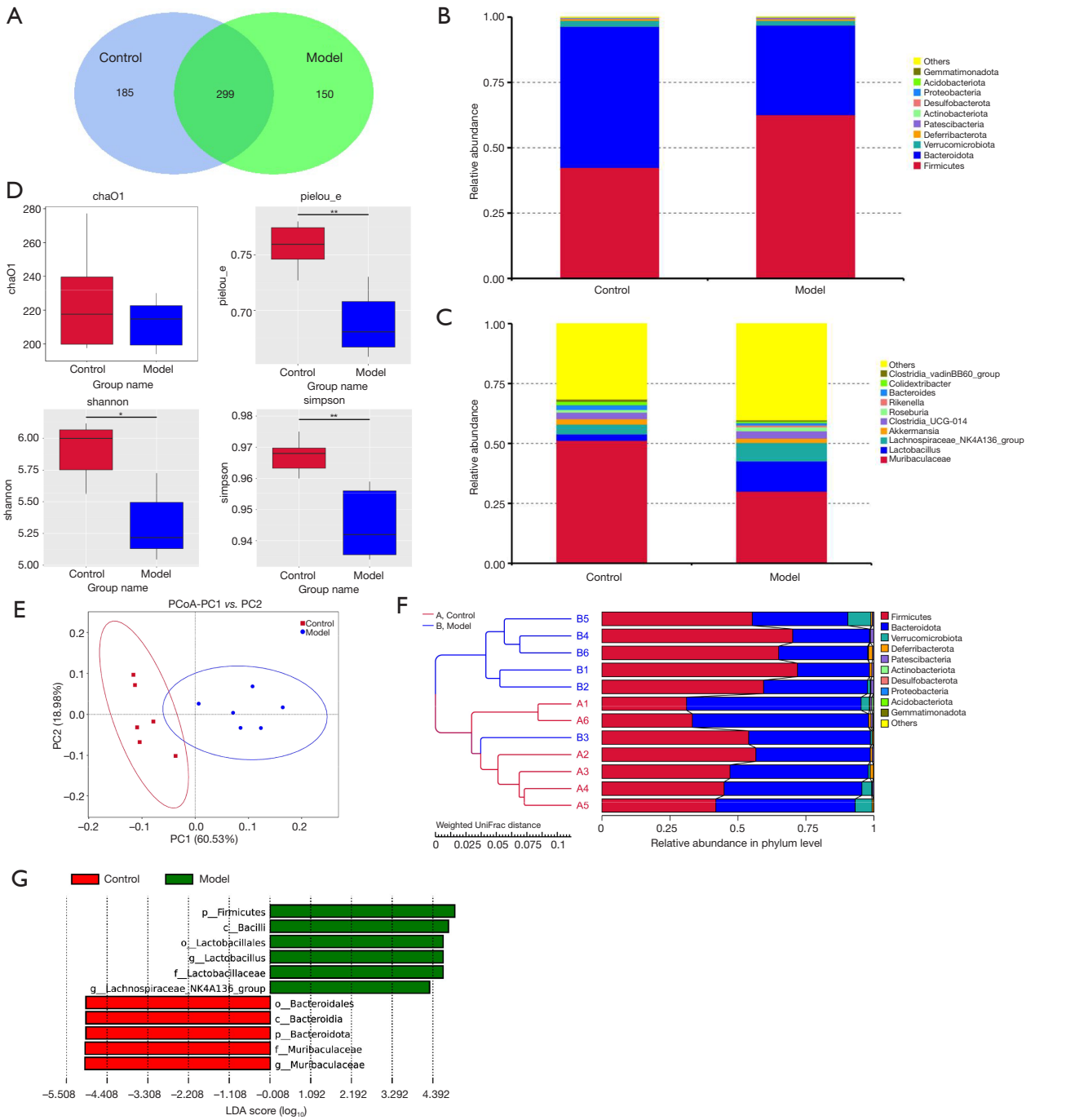

Figure 2 Seawater drowning alters the composition of the intestinal microbiome. (A) Venn diagram of intestinal flora species in two groups. (B,C) Composition and relative abundance of bacterial phylum (B) and genus (C) in two groups. (D) Alpha diversity of bacterial communities. The boxplots show median, quartile, smallest, and largest observations. *, P<0.05; **, P<0.01, respectively, versus the control group. The Wilcoxon rank-sum test was performed to determine statistical significance of alpha diversity analyses. (E) The PCoA plot is generated of the Weighted UniFrac Distance based on OTU counts and explains the largest variance among all samples. (F) Community composition at the phylum level of each sample is shown with hierarchical clustering based on Weighted UniFrac Distance of OTU profiles. (G) LDA effect size analysis between two groups. PCoA, principal co-ordinates analysis; OTU, operational taxonomic unit; LDA, linear discriminate analysis.

The community diversity and richness were calculated using the alpha diversity and beta diversity indicators. As shown in *Figure 2D*, the Pielou-e index, Shannon index and Simpson index indicated that seawater exposure caused a significant reduction in the diversity of intestinal microbiome. Chao1 index did not observe a significant difference between the two groups. The PCoA plot showed that seawater exposure induced a distinct structure of the intestinal microbiome (*Figure 2E*), with the PC1 and PC2 principal components explaining 60.53% and 18.98% of the variation, respectively. Furthermore, the hierarchical clustering analysis using UPGMA demonstrated that most control and seawater-exposed samples clustered in their respective groups (*Figure 2F*).

To confirm the most relevant taxa responsible for the effects of seawater drowning-induced ALI, a LDA of the effect size algorithm was performed with a threshold of logarithmic LDA score >4.0. Seawater-drowned mice had higher abundance of *p_Firmicutes*, *c_Bacilli*, and *g_Lachnospiraceae_NK4A136_group*, whereas *g_Muribaculaceae*, *f_Muribaculaceae*, and *p_Bacteroidota* were more abundant in the control group (*Figure 2G*). These changes in bacterial abundance suggested that seawater drowning leads to the emergence of differential intestinal microbes microecology in mice.

Effect of seawater drowning on gene expression in mice

To gain a deeper understanding of the gene expression profiles in mice affected by seawater drowning-induced ALI, we performed RNA-seq to investigate the effect on transcription. RNA-seq of the 12 grouped samples (6 biological replicates each) yielded a total of 520 Gb clean reads. PCA demonstrated the variability between groups and the similarity within groups (*Figure 3A*). The results of differential expression analysis showed that a total of 4,045 up-regulated and 4,080 down-regulated DEGs were found in ALI mice compared to the negative control (NC) group (*Figure 3B*). Hierarchical clustering heatmaps indicated expression differences between groups and homogeneity within groups (*Figure 3C*). The results showed that the gene expression profile of mice was significantly changed due to seawater drowning. GO and KEGG enrichment analysis of DEGs revealed that they were mainly involved in the biological functions associated with inflammation such as cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor, and chemokine signaling pathway (*Figure 3D*). The GSEA analysis showed the same results as well (*Figure 3E*). These results further suggest that seawater drowning leads to differential gene expression in mice lungs, and that the DEGs are enriched in biological entries associated with inflammation, which in turn suggests that seawater-drowned mice develop inflammation in their lungs, causing lung injury.

Effect of intestinal microbiota on host lung transcriptome profiles through inflammatory responses

Based on the stool microbiota and lung transcriptome data described above, Pearson correlation analysis was performed to identify microbe-associated genes and to test whether transcriptional profiles in ALI mice might be partially influenced by the gut microbiota. A total of 8,124 genes and 5 OTUs were identified by correlation analysis, resulting in 40,625 OTU gene pairs (*Figure 4A*). Through gene annotation and pathway analysis, 1,690 DEGs and 5 OTUs were identified as significantly associated, and 2,645 OTU-gene pairs were formed. Among them, 422 pairs were significantly negatively correlated, involving 361 DEGs. KEGG pathway analysis showed that these DEGs converged in the virus protein interaction with cytokine and cytokine receptor, cytokine-cytokine receptor interaction, IL-17 signaling pathway, chemokine receptor interaction and cytokine receptor interaction signaling pathway, chemokine signaling pathway-related pathways, which were important pathways for inflammation (*Figure 4B*). These further suggested that gut microbiota may influence the host lung transcriptome through common factors.

Seawater exposure activated inflammatory responses and oxidative stress in the lungs

The results of RNA-seq suggested that inflammation occurred in the lungs of seawater drowning-induced ALI mice, and inflammatory factors (IL-1β, IL-6, IL-10 and TNF-α) were selected for further analysis. The expression of inflammatory factors was first extracted from the gene expression profiles by RNA-seq, and it was evident that the expression levels of IL-1β, IL-6, IL-10 and TNF-α were significantly higher in seawater-drowned mice than in the control group, especially IL-6 and TNF-α (*Figure 5A*). ELISA and qRT-PCR results also showed that the expression of inflammatory factors was notably increased in the lungs of seawater-drowned mice (*Figure 5B*,*5C*). Moreover, we detected the levels

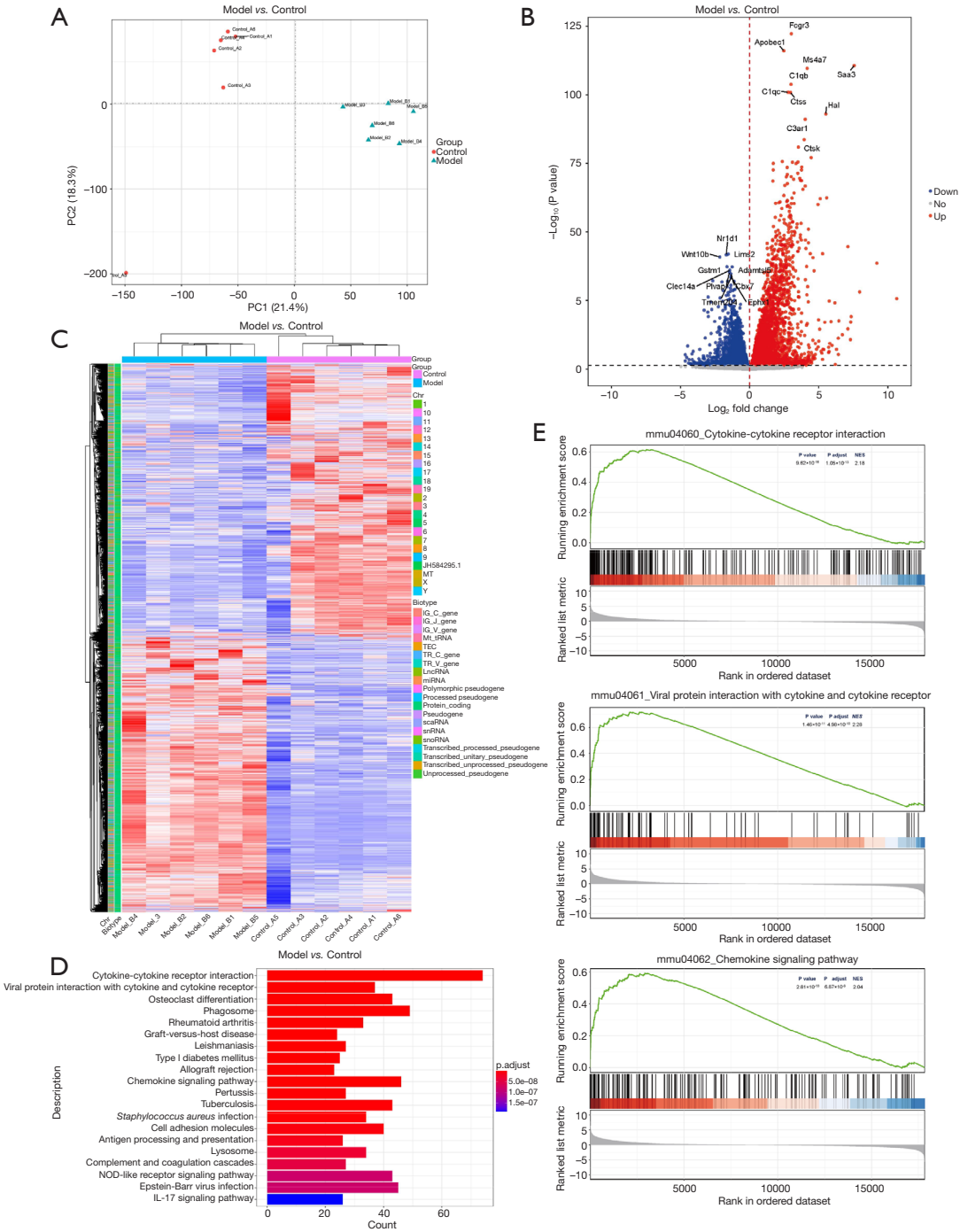

Figure 3 Seawater drowning changes gene expression profiles in mice lungs. (A) Principal component analysis diagram shows the clustering of each group of samples. (B) Volcano plot indicating the differentially expressed gene between two groups. (C) Heat map for hierarchical clustering of differentially expressed genes between two groups. (D) KEGG analysis of differentially expressed genes. (E) GSEA plot of genes involved in inflammation signaling pathways. PC, principal component; IG_C, constant chain immunoglobulin; IG_J, joining chain immunoglobulin; IG_V, variable chain immunoglobulin; Mt, mitochondrial; tRNA, transfer RNA; TEC, to be experimentally confirmed; TR_C, constant chain T cell receptor; TR_V, variable chain T cell receptor; lncRNA, long non-coding RNA; scaRNA, small Cajal body-associated RNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; NES, normalized enrichment score; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, gene set enrichment analysis.

Figure 4 Association analysis of the microbiome and transcriptome. (A) The Pearson correlation heatmap of differentially microbes and differentially expressed genes. (B) KEGG pathway analysis of differentially expressed genes regulating differential microbes. Corr, correlation; KEGG, Kyoto Encyclopedia of Genes and Genomes.

of oxidative stress molecules (SOD and MDA) in serum, and the results showed that seawater drowning induced oxidative stress in the lungs of mice with a decrease in SOD activity and an increase in MDA activity (*Figure 5D*,*5E*). These results suggested that seawater drowning activates inflammatory responses and oxidative stress in mouse lungs, and demonstrated that inflammatory factors play an indispensable biological function that leads to lung injury in mice.

Discussion

Drowning is a global public health and safety issue, and ALI is one of the common complications in patients drowning for which effective treatment is still lacking (20). Therefore, understanding the key mechanisms of seawater drowning-induced ALI and searching for therapeutic strategies remain a tireless endeavor for scientists. Generally, various factors such as inflammation, apoptosis, and oxidative stress are

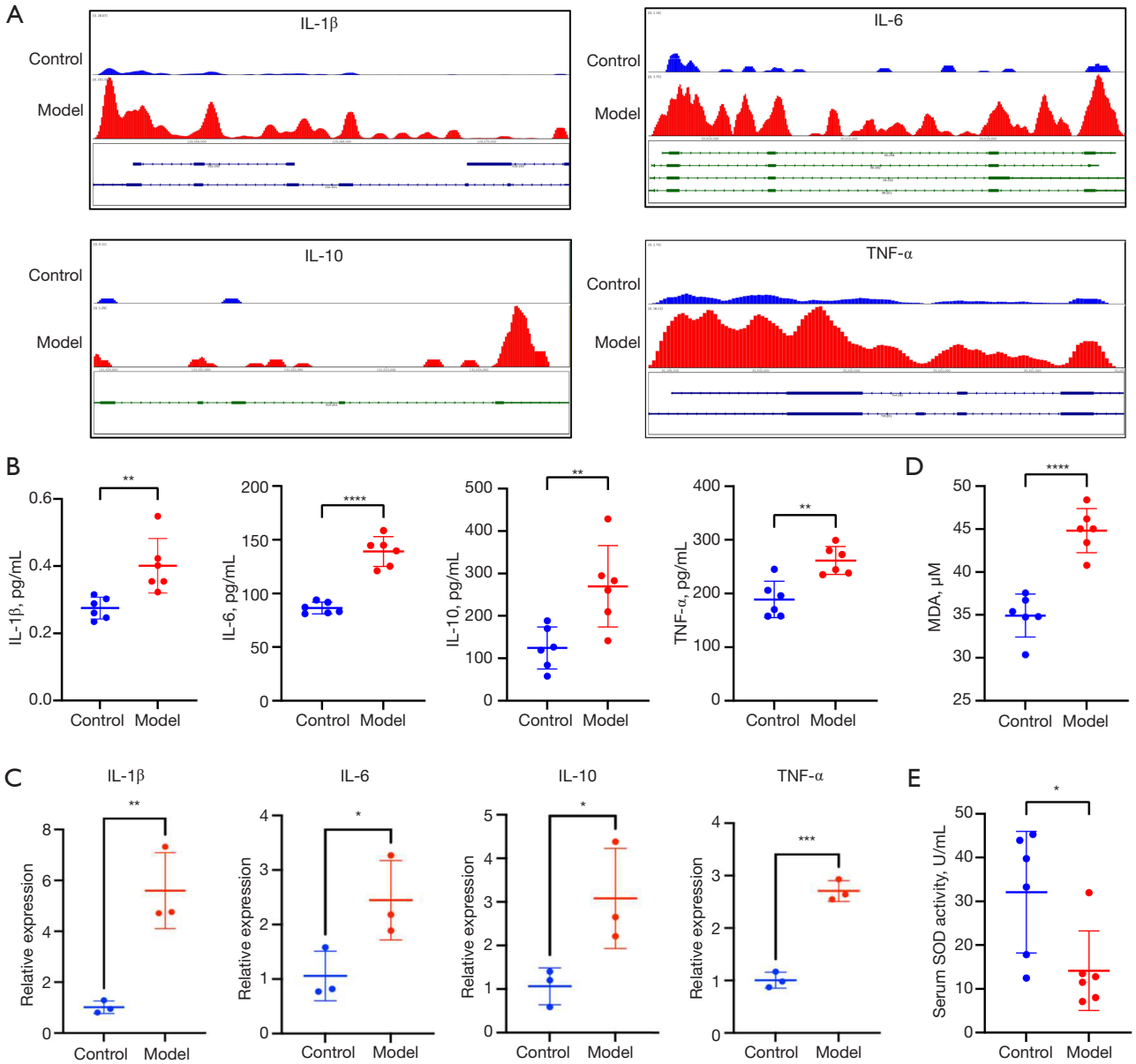

Figure 5 Seawater drowning induces biological responses in the lungs. (A) Transcript expression of inflammatory factors (IL-1β, IL-6, IL-10 and TNF-α) extracted by RNA-sequencing analysis. (B,C) Detection of serum inflammatory factors IL-1β, IL-6, IL-10 and TNF-α by enzyme-linked immunosorbent assay (B) and quantitative real-time polymerase chain reaction (C). (D,E) Detection of MDA (D) and SOD (E) activities in serum. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001, versus the control group. The *t*-test was performed to determine statistical significance. MDA, malondialdehyde; SOD, superoxide dismutase.

closely related to ALI induced by seawater drowning (7). In this study, we demonstrated that seawater exposure directly aggravated pulmonary edema. The landscape of the microbe-gene-lung axis was depicted by combined 16S rRNA and RNA-seq analyses, meanwhile suggesting that inflammatory response is a key contributor in seawater drowning-induced ALI.

Hyperosmotic seawater can directly affect the regulation of lung surface-active substances and alveolar epithelium, influencing alveolar composition by promoting epithelial cell injury and apoptosis, leading to altered barrier function (18). Moreover, the hypertonic nature of seawater

induces direct epithelial cell stimulation and leads to cell shrinkage, which increases the interstitial space between cells, thereby increasing the permeability of these cells (7). Inflammatory cells subsequently infiltrate the alveolar space and release various molecules, leading to worsening of pulmonary edema and gas exchange (21). Many cytokines such as TNF-α and IL-6, are critical in initiating, amplifying and sustaining lung injury (22). These pathological processes can conversely lead to alveolar epithelial-endothelial capillary barrier disruption, pulmonary edema, and severe refractory hypoxemia. In the present study, we confirmed the up-regulation of IL-1β, IL-6, IL-10, and TNF-α expression in the lungs of ALI mice induced by seawater drowning in multiple ways.

Intestinal microbes are important regulators of intestinal homeostasis, of which the composition is closely related to host physiology, and microbial imbalance may lead to various diseases and immune responses (23). *Firmicutes* and *Bacteroidota* are the two most dominant groups in the gut, and the ratio of *Firmicutes* to *Bacteroidota* (F/B) is an important indicator of changes in the structure of the intestinal flora (24). The ratio of F/B was elevated in hyperoxia-induced ALI mice, which was reduced by high fiber diet and dietary supplementation with acetate, thus correcting the intestinal ecological dysbiosis, attenuating ALI (25). An increase in the F/B ratio was similarly observed in LPS-induced ALI mice, along with an increased pulmonary inflammatory response (26). F/B ratios were also increased in patients with inflammatory bowel disease, supporting the notion that F/B ratios are closely related to host inflammation (27). Our data suggested that *Firmicutes* and *Bacteroidota* are the most abundant phylum with elevated F/B ratios in the intestinal contents of seawater drowning-induced ALI mice. Besides, *Lachnospiraceae* was significantly increased in post-infectious cough (PIC) rats, whey protein-sensitized mice, and colon injury mice, correlating with inflammatory responses such as airway inflammation and allergic enteritis to some extent (28-30). Similarly, our results likewise observed that at the genus level, *Lachnospiraceae* was enriched in the seawater-exposed group, which may explain the role of gut flora imbalance in exacerbating ALI. Of course, gut flora undergoes various changes during ALI treatment. The interaction between gut flora and lung disease occurs through the gut-lung axis, and changes in gut flora may affect immune responses and inflammatory processes in the lungs. For example, SCFAs produced by gut flora play a role in modulating gut homeostasis and lung immune responses in mice with LPS-induced ALI, and supplementation with symbiotic can have a beneficial effect on lung health associated with elevated levels of SCFAs (31,32). Supplementation with *Lactobacillus rhamnosus* (LR) significantly improved vascular permeability in the lungs by modulating neutrophils, while significantly decreasing the expression of inflammatory cytokines in the bronchoalveolar lavage fluid, lungs, and serum of mice (33). *Nymphaea candida* (NCTP) has a preventive effect on LPS-induced septic ALI in mice by a mechanism related to the regulation of intestinal flora, SCFA metabolism, and the TLR-4/NF-κB and NLRP3 pathways (34). Taken together, intestinal flora plays an important role in the occurrence, development and treatment of ALI.

To elucidate the underlying molecular mechanisms of ALI induced by seawater drowning, we sequenced the lung transcriptome and analyzed the biological functions and pathways of DEGs. Notably, KEGG analysis revealed that cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor were significantly associated with inflammatory responses. These results were further also illustrated by the association analysis between microbes to genes. A previous study showed that parasite-infected large yellow croaker activates the cytokine-cytokine receptor interaction pathway, exacerbating inflammation (35). Under inflammatory conditions, Wnt inhibitor treatment of human dental pulp stem cells down-regulated KEGG pathways such as cytokine-cytokine receptor interaction and IL-17 signaling pathway (36). Suppression of inflammation through oral administration of intestinal probiotic drugs is a common therapeutic strategy, and one study significantly enhanced the therapeutic efficacy of colitis by utilizing the 3-hydroxybutyrate-producing probiotic *Escherichia coli*, a microbicidal drug that is targeted, slow-release, and controlled (37). Systematic studies of microbe-gene pairs and frontier research on engineered microbial therapeutics can help to further elucidate the development of ALI and provide new targets for the prevention and treatment of ALI.

Moreover, oxidative stress has been recognized as a key pathophysiological feature of lung injury. An abnormal state of oxidative stress accelerates the development of lung injury and may even induce lung cancer. ALI mice usually exhibit an abnormal state of oxidative stress, such as elevated levels of MDA in the lungs (26,38). Studies have suggested that seawater may contribute to oxidative stress in the lungs, which is consistent with our findings (7,39,40). MDA is the end product of peroxidation of polyunsaturated

fatty acids, and when ROS is overproduced, they initiate lipid peroxidation, leading to the production of MDA (26). High concentrations of MDA disrupt cellular integrity through cross-linking and polymerization of proteins or DNA (26). SOD is a key antioxidant enzyme, and superoxide radicals can be converted to H_2O_2 by SOD, which is then degraded to water and oxygen (41). Therefore, reduction in MDA activity and increase in SOD activity are important strategies to protect cells from oxidative stress and may ameliorate lung inflammation. Evidence of hyperoxaluria-induced renal injury in a rat model showed an increase in the ratio of F/B, as well as an increase in the levels of MDA, inflammatory cytokines and inflammatory mediators, and a decrease in the levels of SOD, demonstrating that oxidative stress and inflammatory responses occurred (42). Similar findings were found in the rat model of Gestational diabetes mellitus and the mouse model of alcohol-induced liver injury, suggesting the efficacy of anti-inflammatory and antioxidant therapies in injury diseases (9,43). Taken together, our results suggest that reducing the abundance of *Firmicutes* and enhancing the abundance of *Bacteroidota* can help to alleviate oxidative stress and inflammatory responses in diseased animals.

Conclusions

Seawater drowning is a complex injury process involving pulmonary edema formation, enhanced inflammatory response and oxidative stress. In this study, we observed that seawater exposure aggravated lung injury, increased apoptosis, and enhanced oxidative stress in mice by constructing a seawater drowning mouse model. Microbiome, transcriptome and molecular experiments further confirmed that seawater exposure altered the structure of the gut microbiota in mice, playing a key role in increasing the proportion of *Firmicutes* and *Bacteroidota*. Significant roles were found on inflammation-related pathways with increased expression of inflammation-related factors. This study depicts the landscape of the microbe-gene-lung axis contributing to further understand the mechanism by which alterations in intestinal microbial structure activate inflammatory signaling pathways that exacerbate ALI, thus providing new strategies for clinical applications of microbes to intervene in the control of ALI.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-633/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All experiments were performed in accordance with relevant laws and institutional guidelines for the care and use of animals, and approved by the Laboratory Animal Welfare and Ethics Committee of the Army Medical University (No. SYXK (YU) 2022-0018), in compliance with the national or institutional guidelines for the care and use of animals.

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