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**The Time-dependent dynamics of gut microbiota and the interaction with host
metabolism in mouse pulmonary fibrosis models**

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Abstract

Background: Pulmonary fibrosis (PF) is a chronic progressive disease whose pathogenesis is thought to be associated with activation of the immune system and consequent metabolic changes. Recent studies suggested that gut microbes are closely related with host's immune response and metabolic changes in fibrotic hosts. However, the dynamic changes of the gut microbiome and the interaction profiles with host metabolism during the development of pulmonary fibrosis remain inconclusive.

Results: We collected serum and fecal samples from bleomycin-induced fibrotic mice at 0, 7, 14, and 28 days and performed UPLC-MS analysis on serum metabolites and metagenomic sequencing on fecal samples. It is found that the serum metabolic profile and gut microbiome were significantly altered in mice during the progression of fibrosis. Among the serum metabolites, the levels of three major types of lipids, *i.e.*, glycerolipids, glycerophospholipids, and fatty acids exhibit significant time-dependent changes.

The glycerolipid TG and multiple glycerophospholipids (3 PG, 6 PE, and 1 PC) decreased in the early stage of fibrosis and increased in the late stage. The other two types of glycerolipids MG and DG and the fatty acids Cartinine and Punicic acid decreased through the development of fibrosis. In the meantime, we detected significantly elevated abundance of gut microbiome taxa, including *Prevotella sp.* from Bacteroidetes, *Lactobacillus* from Firmicutes, and *Bifidobacterium* from Actinobacteria in mice with pulmonary fibrosis. When compared to the dynamic profiles of serum metabolites, the abundances of gut microbiome show a high level of

correlation with that of serum metabolites. The taxa from *Bacteroides*, such as *Butyricimonas_synergistica* and Muribaculaceae, show positive correlation with the cluster of glycerophospholipids, while taxa from *Firmicutes*, such as *Clostridioides difficile* and *Enterococcus faecium* exhibit negative correlation. Further functional classification suggested that those taxa are involved in multiple functional modules, such as Transporters, Secretion system, and Metabolism.

Conclusions: The results reveal the synergistic changes between the gut microbiome and host metabolism and the dynamic responses of gut microbiome to host fibrosis during the progression of fibrosis.

Keywords: Pulmonary fibrosis, gut microbiota, serum metabolites, metagenome, metabolic disorders, mice

INTRODUCTION

Pulmonary fibrosis (PF) is a chronic progressive disease with excessive deposition of collagen tissue in the lung interstitium. It is a common and irreversible interstitial lung disease and one of the serious diseases of the respiratory system [1]. The patients would develop lung fibroblast proliferation, extracellular matrix collagen deposition, airway inflammatory cell infiltration, etc., which eventually led to alveolar and lung tissue fibrosis [2]. Due to the lack of reliable early diagnosis methods and effective treatment methods to reverse the natural process and outcome of fibrosis, the median survival time from diagnosis was only 2.5-3.5 years, and the 5-year survival rate was less than 40% [3, 4]. Pulmonary fibrosis may be even more malignant than some malignant tumors because of progressive respiratory failure.

Pulmonary fibrosis is usually caused by different stimuli such as toxin, infection, severe trauma, autoimmune reaction, adverse drug reaction, and idiopathic, unknown factors [5, 6]. The pathogenesis of pulmonary fibrosis is not completely clear. At present, it is believed that the development of pulmonary fibrosis can be divided into two stages, namely the early stage of alveolar inflammation and the late stage of excessive fibrotic repair. In the early stage of alveolar inflammation, the pulmonary activated interstitial and infiltrating cells will secrete a series of cytokines, including tumor necrosis factor (TNF- α), chemokines (CXC), and adhesion cytokines (CAM), which affect each other and promote further aggregation of inflammatory monocytes; in the later stage of fibrosis, inflammatory cells and interstitial cells (fibroblasts and myofibroblasts) were activated to secrete factors such as growth factor, interleukin (IL) and matrix metalloproteinase (MMPs), which were involved in the repair and remodeling of lung tissue and blood vessels, and eventually led to the occurrence of pulmonary fibrosis [7-9].

In the process of pulmonary fibrosis, a series of metabolite changes occurred. Pulmonary fibrosis can lead to glycolysis reprogramming. Inhibition of mouse glycolytic enzyme 6-fructose phosphate-2-kinase/fructose-2 by pharmacological or genetic methods, 6-Diphosphatease 3 (PFKFB3) not only reduced fibroblast activation in vitro but also significantly improved the severity of pulmonary fibrosis [10]. Subsequent studies confirmed that this metabolic reprogramming was closely related to macrophage activation, and the M2 macrophage distribution of fibrosis in fibrotic alveolar was independent of fatty acid oxidation and synthesis or lipolysis but

relied on glycolytic [11].

With the development of pulmonary fibrosis, the metabolome of the organism is also altered. Sung-Woo Park *et al.* analyzed lung tissue using mass spectrometry and identified 25 altered metabolites associated with pulmonary fibrosis, mainly involved in metabolic pathways such as the adenosine triphosphate degradation pathway and the glycolytic pathway [12]. Cédric Bovet *et al.* tested serum from patients with pulmonary fibrosis, finding that LysoPC has the potential of serving as a biomarker for pulmonary fibrosis [13].

A variety of evidences show that some diseases can cause changes in gut microbiota, and meanwhile, gut microbiota plays a role in immune protection against lung diseases (such as pneumonia and asthma) [14, 15]. Marie-Clair Arrieta *et al.* analyzed the metabolites of the feces and urine of infant mice of different ages, and demonstrated that the composition and abundance of the gut microbiota at the age of 3 months affect the risk of human asthma [16]. Its main research methods included 16S rRNA gene sequencing and whole-genome sequencing. Jin Su *et al.* studied the feces of mice with pulmonary fibrosis using metabolomic and 16S rDNA sequencing techniques, revealing a previously unknown link between the intestine and pulmonary fibrosis [17]. Although adverse changes in the intestinal ecosystem have been identified in multiple diseases, such as asthma, lung cancer, and acute lung injury, the details about the intestinal microbiome and the interaction between the microbiome and host metabolism in pulmonary fibrosis models have not yet been fully elucidated. [16, 18-21].

In the present study, we aimed to examine the interactions between the gut microbiota and serum metabolome, and investigate their dynamic changes in mice with pulmonary fibrosis using multi-omics approaches.

MATERIALS AND METHODS

Animal, Fibrotic Models and Sample Collection

All experiments were approved by the Ethics Committee of Affiliated Hospital of Qingdao University (Ethics NO. QYFYW2LL26275). Specific-pathogen-free C57BL/6 J male mice (about 8 weeks of age) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.

All mice were housed in a barrier facility and were fed normal-chow diet. They were randomly assigned to the four groups (control, model_7Day, model_14Day, model_28days) with 10 mice in each group. To induce pulmonary fibrosis, mice were injected intraperitoneally and anesthetized suitably with 1% pentobarbital sodium, and then vertically mounted on a slope. A laryngoscope was held at the throat of the mice and the tongue was pulled lightly, a hermetic venous indwelling needle was plugged in and the inner tube was taken out, the drug (2.0 mg/kg bleomycin for experimental group, the same amount of normal saline for normal group) was dropped into the trachea, the tube was unplugged and the mice laid in a mouse cage.

Lung tissue, serum, and fecal samples were collected for histopathological, metabolomic, and metagenomic assays from drug-induced mice at 7, 14, and 28 days as well as from control mice.

Pathological Analysis and Immunohistochemistry

Mice were deeply anesthetized with sodium pentobarbital and died from blood loss after incision of the abdominal aorta. After induction of pulmonary fibrosis, the heart was perfused with PBS and the left lobe was injected with approximately 250 μ l 4% paraformaldehyde, and then the 4% paraformaldehyde-fixed tissue was routinely processed, embedded in paraffin, and sectioned. Two adjacent slices were stained with TGF- β , HE, and Masson, respectively, and the stained slides were scanned with PANNORAMIC DESK/MIDI/250/1000 (3DHISTECH, Hungary), and representative slice areas are shown in the screenshots.

UPLC-MS Metabolome Profiling

Metabolite extracts were obtained from serum samples following methanol-assisted protein precipitation and then analyzed using an LC-ESI-MS/MS system (UPLC, ExionLC AD, <https://sciex.com.cn/>; MS, QTRAP® System, <https://sciex.com/>). Liquid phase separation was performed using a Waters ACQUITY UPLC HSS T3 C18 column (1.8 μ m, 2.1 mm*100 mm). LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole linear ion trap mass spectrometer (QTRAP) controlled by Analyst 1.6.3 software (Sciex) QTRAP® LC-MS/MS system. A specific set of MRM shifts was monitored for each period based on the metabolite elution in each period. Software Analyst 1.6.3 was used to process the mass spectrometry data. Several existing mass spectrometry public databases were consulted for metabolite structure analysis, mainly massbank (<http://www.massbank.jp/>), knapsack (<http://kanaya.naist.jp/knapsack/>), HMDB

(<http://www.hmdb.ca/>) and Metlin (<http://metlin.scripps.edu/index.php>). Qualitative analysis was performed based on retention times and mass-to-charge ratios of parent and daughter ions of test substances from internal and other public databases. The quantitative analysis was performed by the triple quadrupole mass spectrometry multiple reaction monitoring (MRM) method. The signal intensity of the characteristic ions is obtained in the detector. MultiQuant is used to integrate and calibrate the chromatographic peaks. The peak area of each chromatographic peak represents the relative amount of the corresponding substance[22, 23].

The Kruskal–Wallis H test was used to determine the differences between groups. The lipidomics data were normalized using R software (<https://www.r-project.org/>). SIMCA 14.1 was used to analyze the metabolites using principal component analysis (PCA) and orthogonal partial least squares discrimination analysis (OPLS-DA) model. VIP>1 and P values <0.05 were considered significant. Asterisks represent: ns means no significant, *P < 0.05, **P < 0.01, *** P < 0.001 and **** P < 0.0001.

DNA Isolation and Metagenomic Sequencing and Assembly

Total genomic DNA was extracted from fecal samples using the Mag-bind Soil DNA Kit (Omega Biotek, USA) according to the manufacturer's instructions. Approximately 2 µg of DNA was prepared per sample. Sequence libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA). The libraries were sequenced on the Illumina NovaSeq /HiSeq platform (insert size 350 bp, read length 150 bp) at the Novogene Bioinformatics Technology Co., Ltd. (Tianjin, China). FastQC is used to assess the quality of raw data comprehensively

generated by sequencing, while we screened the data for quality, removing sequences less than 50 bp in length or containing ambiguous bases. All metagenomic raw data have been submitted to the NCBI database (accession: PRJNA722397).

Gene Catalog (Unigenes) Construct and Taxonomy Predication

Prediction of genes using MetaGeneMark version 2.10 (<http://exon.gatech.edu/GeneMark/>) [24]. Redundant genes (95% identity, 90% overlap) were removed by MMseqs2, resulting in a non-redundant gene catalog. Taxonomic function assignment and taxonomic identification of the readings using MEGAHIT. The LCA algorithm of the MEGAN software system was used for annotation.

Gene Functional Annotations

Predicted unigenes were assigned to the Kyoto Encyclopedia of Genes and Genomes (KEGG) using KOBAS, the Carbohydrate-Active Enzyme Database (CAZy), and the Gene Evolutionary Lineage: Unsupervised Orthologous Group (eggNOG) using Diamond and the Carbohydrate-Active Enzyme Database (CAZy) using eggnog-mapper. The abundance of each functional annotation is the sum of the abundance of each functional level annotation.

Correlation Analysis of Gut Microbiota and Metabolites

To determine the association between gut microbiota and metabolites in the pulmonary fibrosis model, we constructed a correlation analysis between gut microbiota and differential metabolites using Spearman's correlations in R version

3.4.3 (Hmisc package). The first 52 species and metabolites between the four groups were analyzed.

Data Analysis

To identify features (taxa and functional modules) differentially represented between the four groups, differential abundance taxa or functional modules were selected using LEfSe (linear discriminant analysis (LDA)) effect sizes. Differential abundance of phylum, genus, species and functional modules between any two groups was tested by metagenomeSeq [25], and P values were corrected for false discovery rate (FDR) using the Benjamini-Hochberg method.

RESULTS

The Establishment of Pulmonary Fibrosis Mouse Models Induced by Bleomycin

We used bleomycin-induced mice to construct a pulmonary fibrosis model and collected their lung tissues, serum, and feces at four time points, *i.e.*, 0 day (control), 7th day (Model_7Day), 14th day (Model_14Day), and 28th day (Model_28Day) (Figure 1A). No significant changes in body weight and dietary intake were observed in bleomycin-induced mice as compared to the control group ($p>0.05$). TGF- β western blot and immunohistochemistry of mice lung tissues showed that there exist typical and large fibrotic lesions in the models (Figure 1B and Figure 1D), although TGF- β secretion of lung tissues decreased in 28 days. Immunohistochemistry on lung tissue sections in mice further demonstrated that bleomycin induced strong fibrosis in the lungs of mice (Figure 1C). Together, the above data demonstrated that the serum

and stool samples in the model were reliable for further gut microbiota sequencing and metabolomic assays.

The Altered Metabolism in Serum of Mouse Fibrosis Models

The time-series metabolic profile in the serum of the mouse fibrosis models was obtained using mass spectrometry (MS) assays. Clustering analysis based on PCA and OPLS-DA showed clear separation between the control and fibrosis models, indicating the metabolic shift in the fibrosis models as compared to the control (Figure 2A). A total of 585, 626, 141 unique metabolites are differentially represented in the Model_7Day, Model_14Day, Model_28days, respectively in comparison with the control ($VIP > 1$ and $p\text{-value} < 0.05$) (Table S1). 49 top metabolites significantly changed in all three fibrotic models are presented in the hierarchical clustering heatmap (Figure 2B and Table S2). It is shown that those metabolites form four clusters (C1-C4) and are dominated by three types of lipids, i.e., glycerophospholipids, glycerolipids, and fatty acids. Glycerophospholipids constitute the majority of the cluster C2 (including 3 PG, 6 PE, and 1 PC) and exhibit a time-dependent pattern of decreasing in the early stage of fibrosis (Model_7Day and Model_14Day) and increasing in the late stage (Model_28Day), while both glycerolipids and fatty acids fall into multiple clusters with divergent time-dependent patterns. For example, the tri-radyl type of glycerolipid TG clusters together with glycerophospholipids in C2 by sharing similar time-dependent pattern, whereas the duo-radyl and mono-radyl type of glycerolipid (MG and DG) falls into the cluster C4, where the abundances of the metabolites decreased through the development of fibrosis. The fatty acids are

scattered in the cluster C4 (such as Cartinine and Punicic acid) and C1 (such as Linoleic acid, Hexadecanoic acid, Elaidic acid, and 8,11-icosadienoic acid). In addition, the nucleotide Adenosine is special in that its abundance is only increased in the Model_14Day, distinguished from other metabolites.

Further pathway enrichment analysis identified five pathways (glycerophospholipid metabolism, glycerolipid metabolism, linoleic acid metabolism, sphingolipid metabolism, and Arginine biosynthesis) enriched with the differentially represented metabolites across the three time points, indicating the relationship between pulmonary fibrosis and aberrant metabolism of the major lipids in serum, such as glycerophospholipid, glycerolipid, fatty acids, and sphingolipid (Figure 2C-2D).

Alterations in Gut Microbial Compositions in the Mouse Fibrosis Models

To evaluate the changes of intestinal microbes in the process of lung fiber formation in mice, we performed metagenomic testing on the feces of mice. The optimized reads ranging from 74,230,682 to 99,459,116 were obtained from all samples. The average quality distribution of sequences showed a peak width of 36 for the quality Q-value distribution of sequencing data, indicating high sequencing quality. After data quality control, a high percentage of valid sequences (~99%) was achieved in all samples. This indicates that the sequencing depth was sufficient for the investigation of the fecal microbiota.

The taxonomic abundance analysis showed that the gut microbiota of the mouse models was dominated by families from *Firmicutes*, *Bacteroidetes*, *Proteobacteria*,

264 and *Actinobacteria*. There are no significant differences in the taxonomic diversity
265 among the four groups based on the alpha diversity calculation (Shannon,
266 Kruskal-Wallis test, $p = 0.2075$) (Figure 3A). Principal coordinates analysis (PCoA)
267 showed that the Model_7Day and Model_14Day are closer to each other, while the
268 control and Model_28Day gathered together, with the first (PC1) and second (PC2)
269 principal coordinate accounting for 19.9% and 14.9% of the total variation,
270 respectively (Figure 3B). In order to identify the discriminating taxa in each of the
271 four mouse groups, we applied LEfSe (LDA Effect Size) analysis to the samples and
272 identified 79 taxa with differential abundance across the four groups (Table S3). Their
273 discriminating ability indicated as LDA (linear discriminant analysis) score in Figure
274 3C revealed that the control group is characterized by a high abundance of
275 Verrucomicrobiae (such as *Akkermansia muciniphila*), the Model_7Day is enriched
276 with phylotypes from Bacteroidetes (such as *Prevotella* sp.) and Proteobacteria (such
277 as *Pandoraea sputorum*), the Model_14Day is enriched with phylotypes from
278 Firmicutes (such as *Lactobacillus*), while the Model_28Day contains abundant
279 species from Actinobacteria, Bacteroidetes and Proteobacteria. It is seen that although
280 the overall diversity is no significantly different between the four groups of mice, the
281 abundance of specific taxa has altered during the progression of fibrosis. Notably,
282 several phylotypes from Verrucomicrobiae including *Akkermansia muciniphila* have a
283 LDA score as high as 4.5 in the control group, suggesting significant reduction of this
284 phylum during the progression of fibrosis. *Akkermansia muciniphila* has been
285 proposed as a biomarker for a healthy gut and shown to link to intestinal health

(<https://pubmed.ncbi.nlm.nih.gov/23671105/>). Bacteroidetes and Firmicutes are known key players in gut microbiome for regulating host metabolism and their altered abundance has been proposed to be associated with multiple host immune diseases (<https://www.nature.com/articles/s41385-020-0296-4>; <https://pubmed.ncbi.nlm.nih.gov/32887946/>).

Functional Dynamics in Gut Microbiota in the Mouse Fibrosis Models

To further elucidate the functional alterations in the gut microbiota of the mouse fibrosis models, we classified the gut microbiome genes into functional categories and compared the abundance of the categories across the four groups of mice. PCoA based on KEGG module abundance revealed a clear separation between Control and Model_7Day/Model_14Day, while the difference between Control and Model_28Day was relatively small, consistent with the clustering pattern inferred from the taxonomic composition (Figure 4A). Analysis of Similarities (ANOSIM) using KEGG orthologous (KO) terms indicated that the abundance of KO categories are significantly differentiated among the four groups ($p = 0.001$). We presented the top 22 in Figure 4B, including K18197 (rhamnogalacturonan endolyase), K08095 (carboxylic-ester hydrolase), K0771 (AP endonuclease 1), and K16150 (glycogen synthase) among others. A large proportion of the KO categories have elevated abundance in the Model_7Day and/or the Model_14Day.

To unravel the alterations of the biological processes underlying the gut microbiome during the course of mouse fibrosis, we mapped the KO terms to KEGG pathways and compared the pathway abundance among the four groups (Figure 4C

and 4D). It is shown that the altered pathways are mainly involved in “Metabolism”, “Genetic Information Processing”, and “Cellular Processes”, such as Metabolism of other amino acids, Energy metabolism, Lipid metabolism, Glycan biosynthesis and metabolism, Translation, Transport and catabolism, Cell motility, and Cell community. LEfSe analysis further identified the most representative pathways in each stage of mouse fibrosis, where the Model_7Day is characterized by the pathways of Energy metabolism, Model_14Day contains abundant pathways of Cell community (LDA score > 2.5), and Model_28Day is enriched with pathways related to Translation (Ribosome, LDA score > 3), Glycan biosynthesis and metabolism (LDA score > 3.5), and Lipid metabolism (Terpenoid backbone biosynthesis, LDA score > 2.5) (Figure 4E and Table S3). These remarkable changes in pathways in gut microbiome in mouse fibrosis models highlight the link between the fibrosis progression and functional dynamics of gut microbiota and the multi-faceted functional responses of gut microbiome to host fibrosis.

Correlation between gut microbes and serum metabolites

To explore the potential relationships between gut microbiota and serum metabolites, we calculated the abundance correlation matrix between the microbiota taxa and serum metabolites (Figure 5). The correlation matrix between gut microbiota taxa and serum metabolites is self-organized to two separate clusters. The first cluster is dominated by taxa from *Bacteroidetes* (such as *Bacteroides_stercoris*, *Paramuribaculum_intestinale*, and *Butyricimonas_virosa*). Their abundance is positively correlated with the level of diverse GP and two fatty acids Carnitine and

9,12-octadecadienoic acid (called meta-group-1), while negatively correlated with the level of three other metabolites, *i.e.*, L-Thyroxine, cis-1-Pentadecenoic acid, and N-Methyltryptamine (called meta-group-2). In the contrary, the second cluster mainly comprises of taxa from Firmicutes (such as *Clostridioides difficile* and *Enterococcus faecium*) and their abundance correlation with the metabolites exhibits opposite profiles (Figure 5A).

Notably, the correlation matrix between gut microbiota KO categories and serum metabolites is also stratified to two clusters, of which the hierarchical clustering pattern regarding to metabolites is highly in parallel to that for the correlation matrix between gut microbiota taxa and serum metabolites (Figure 5B). Specifically, the first cluster includes two KO terms (K10771- AP endonuclease 1 and K16150-glycogen synthase), which are positively correlated with the meta-group-1. The other 20 KO terms form the second cluster, which are negatively correlated with the meta-group-1 and positively correlated with the meta-group-2. Those 20 KO terms are involved in multiple functional modules, such as Transporters, Secretion system, and Metabolism, nonetheless, none of the modules are enriched with the KO terms. The correlation between gut microbiota functions and serum metabolites reveals synergistic dynamic changes between gut microbiota and host metabolism during the progression of fibrosis. The relevant functions associated with the gut microbiota are diverse implying the complexity of the interactions between gut microbiota and host fibrosis.

DISCUSSION

To understand the time-dependent dynamics of gut microbiota and the interaction with host metabolism in pulmonary fibrosis, we investigated the changes of serum metabolites and gut microbiome in the mouse fibrosis models during the development of fibrosis. We summarized the main findings in Figure 6. Briefly, the serum metabolic profile and the composition of gut microbes were significantly altered in mice during the progression of fibrosis. The alterations at the two loci exhibit a high level of correlation suggesting a potential link between the serum metabolism and the gut microbiota.

We found that the levels of multiple types of glycerolipids and glycerophospholipids began to decrease significantly after one week of induction and did not return to normal levels until the fourth week. These lipids may help to lubricate the cells of the inner layers of the lung and participate in initiating the repair of damaged tissues leading to the production of pulmonary fibers [26-28]. Abnormal repair after alveolar injury leads to the proliferation of fibrous tissue, which involves complex pathological processes and changes in various lipid metabolites [29-31]. Kuroda *et al.* found that changes in phospholipid levels in alveolar lavage fluid of patients with pulmonary fibrosis were closely related to the destruction of alveoli and surfactants and the progression of pulmonary fibrosis [32]. Feng Yan *et al.* showed that the expression levels of glycerophospholipids, which have diagnostic values, were significantly reduced in patients with pulmonary fibrosis by plasma assays [31]. We also found that serum concentrations of glycerophospholipids were significantly reduced in the early stages of the bleomycin fibrosis model. The reduced

glycerophospholipids might be due to their transport into the alveoli and oxidization to generate increased vascular permeability. This not only enhanced lung epithelial cell injury but also further recruited fibroblasts to reach the site of injury. In the late stage of the model, serum glycerophospholipids gradually recovered as vascular permeability decreased.

Adenosine is an endogenous purine nucleotide that mostly regulates cyclic adenosine monophosphate (cAMP) levels through adenosine receptors (ARs) and exerts the corresponding physiological effects [33]. In studies of chronic obstructive pulmonary disease and mouse models of lung disease, significantly increased levels of adenosine were observed [34, 35]. In a bleomycin-induced pulmonary fibrosis model, increased cAMP levels inhibited the overexpression of endothelin in lung tissue and alleviated the symptoms of pulmonary fibrosis, which can directly influence the role of ARs in pulmonary fibrosis [36]. Meanwhile, ARs can induce lung fibroblast differentiation, which is closely related to the release of endothelin-1 and IL-6 from alveolar macrophages and affects the degree of pulmonary fibrosis in mice [37]. In our study, adenosine levels in the model group increased significantly at Model_14Day and started to decrease at Model_28Day, suggesting that adenosine is likely involved in the formation of lung fibrosis in mice and is closely related to the inflammatory response in the early stages of lung fibrosis. Meanwhile, correlation analysis showed that adenosine was positively correlated ($r>0.5$) with *Muribaculaceae_Bacterium_Isolate-114_(HZI)*, which may be involved in the absorption and transformation of adenosine in the intestinal tract.

To the best of our knowledge, gut microbes are associated with the development of a variety of diseases (e.g., lymphoma, liver cancer, breast cancer & pneumonia), but the alteration of gut microbiota in pulmonary fibrosis has rarely been reported [14, 38-43]. Although animal models of fibrosis do not exactly recapitulate IPF, they have yielded many targets for therapeutic intervention [44, 45]. Gen-Cheng Gong *et al.* performed 16S sequencing of gut microbes in a mouse model of pulmonary fibrosis and showed that seven microbes were significantly altered in the pulmonary fibrosis model, including *Alloprevotella*, *Helicobacter*, *Rikenella*, and *Rikenellaceae RC9* showed a decreasing trend, and *Dubosiella*, *Olsenella*, and *Parasutterella* showed an increasing trend [17]. Wenjun Li *et al.* found that in a mouse model of radiation-induced pulmonary fibrosis, the intestinal microbiota was disturbed and intervention to normalize the gut microbiota attenuated pulmonary fibrosis [46]. Ruixue Huang *et al.* examined the gut microbiota of 18 silicosis patients with severe pulmonary fibrosis and confirmed that Firmicutes and Actinomyces bacteria were significantly reduced in patients with silicosis and progressive pulmonary fibrosis compared to healthy individuals [20]. Here, we detected significantly elevated abundance of *Prevotella sp.* from Bacteroidetes, *Lactobacillus* from Firmicutes, and *Bifidobacterium* from Actinobacteria in the intestine of mice with pulmonary fibrosis. Multiple taxa from *Bacteroides*, such as *Butyricimonas_synergistica* and Muribaculaceae, show positive correlation with the cluster dominated by glycerophospholipids, while taxa from *Firmicutes*, such as *Clostridioides difficile* and *Enterococcus faecium* exhibit negative correlation. These results suggest, to some

418 extent, that certain gut microbes were altered in parallel with the serum metabolites
419 during the development of pulmonary fibrosis and are likely to be involved in the
420 pathogenesis of the disease, while the specific mechanism of action between
421 pulmonary fibrosis and gut microbes requires subsequent in-depth studies.

422 By comparing the differential metabolites and microorganisms in Model_7Day,
423 Model_14Day, and Model_28Day with the control group, we found that the number
424 of differential metabolites and microorganisms detected in Model_7Day and
425 Model_14Day was higher than that in Model_28Day. We believe that this
426 phenomenon should be closely related to the pathogenic process of pulmonary
427 fibrosis. At the beginning of the lung fibrosis model, due to the presence of harmful
428 substances, lung fibroblasts were activated and secreted a large amount of
429 extracellular matrix, and the subsequent production of cytokines mediated the
430 proliferation and differentiation of fibroblasts by macrophages approaching the
431 damaged site [1, 8]. As a result, a large number of differential metabolites associated
432 with inflammation appear in the serum during this period. In addition, the gut
433 microbiota is closely related to the immune status of the host [47, 48]. In the later
434 stages of pulmonary fibrosis, the lungs are in a state of the tissue and vascular repair
435 and remodeling, the inflammatory response decreases, the inflammation-related
436 metabolites present in the serum gradually decrease, the fibrosis-related metabolites in
437 the lung tissue begin to increase, and the gut microbiota gradually returns to normal,
438 but still maintains some of the characteristics of the pulmonary fibrosis period.

The advantage of this study is that we applied metagenome sequencing and metabolomics technology, which allowed us to obtain the information about the host-gut microbiota-metabolism interaction in response to the development of pulmonary fibrosis. The limitation of this study is the small sample size, and therefore, further large cohorts are needed to confirm the alterations and to determine the causal relationship between gut microbiota and serum metabolites in human samples. Despite these limitations, the present study opens new avenues for understanding the association between gut microbiota and serum metabolites in pulmonary fibrosis models.

Consent for publication

Not applicable.

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Conflicts of interest

No conflicts of interest, financial or otherwise, are declared by the authors. Author Hui Zheng was employed by the company Wuhan Metware Biotechnology Co., Ltd.. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and supplementary files..

Ethics approval and consent to participate

All experimental procedures and methods were carried out in accordance with the Guide for the Care and Use of Laboratory Animals provided by the institutional Animal Care and Use Advisory Committee for Qingdao University. The study was carried out in compliance with the ARRIVE guidelines. All animal experimental protocols used in this study were approved by the Ethics Committee of Affiliated Hospital of Qingdao University (Ethics NO. QYFYW2LL26275).

Authors' contributions

X.H.Y., X.J.J., and X.J.J. conceived and designed research; X.H.Y, X.S.C., X.M.W., and Y.Z.Z. performed experiments; X.H.Y., F.F.W., J.P.C., H.Y.W., and Y.Z.Z. analyzed data; X.H.Y., H.Y.W, H.Z., and Y.Z.Z. interpreted results of experiments; X.H.Y., X.S.C., F.F.W., H.Y.W., and Y.Z.Z. prepared figures; X.H.Y., X.J.J., X.M.W., J.P.C., H.Z., and Y.Z.Z. drafted manuscript; X.H.Y., X.J.J., H.Z., and Y.Z.Z. edited and revised manuscript; X.H.Y., X.J.J., and Y.Z.Z. approved final version of the manuscript.

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FIGURE LEGENDS

Figure 1 Bleomycin mouse models.

(A) Flow chart of Bleomycin mouse models, collecting feces and serum from 7th

day, 14th day to 28th day after pulmonary fibrosis induced; (B) TGF- β and α -SMA expression of mouse lung tissues at 7, 14, and 28 days after bleomycin instillation; (C) Lung histopathology after a single dose of bleomycin treatment (HE and Masson staining). Representative hematoxylin- and eosin-stained (a, b, c, and d) and Masson's trichrome-stained (e, f, g, and h) lung tissue sections are shown. The mice were sacrificed at 7 (b and f), 14 (c and g), and 28 (d and h) days after bleomycin instillation. Except for the PBS control (a and e), all panels showed lung sections from bleomycin-treated mice. The lungs were inflated with 10% buffered formaldehyde. Original magnification, 5.0X; insets: 20X; (D) The results of TGF- β immunohistochemistry. Positive Area Ratio, reflecting the proportion of the positive area.

Figure 2 Metabolome profiling of serum.

(A) The multivariate statistical model of the Control, Model_7Day, Model_14Day, and Model_28Day groups. Principal component analysis (PCA) of Control, Model_7Day, Model_14Day, and Model_28Day ($R^2X=0.617$, $Q^2=0.409$); The clustering analyses of orthogonal partial least-squares discriminant analysis (OPLS-DA) of Control and Model_7Day group ($R^2X=0.614$, $R^2Y=1$, $Q^2=0.944$), Control and Model_14Day group ($R^2X=0.59$, $R^2Y=1$, $Q^2=0.928$), and Control and Model_28Day group ($R^2X=0.349$, $R^2Y=0.99$, $Q^2=0.527$). (B) Relative abundance of representative differential serum metabolites in three groups, as compared to that of respective Control group. GP, glycerophospholipids; GL, glycerolipids; FA, fatty

acyls;(C) KEGG pathway analysis, the color of the point represents the *p*-value, the size of the point represents the enrichment factor, and the shape of the point represents the changes in metabolic pathways; (D) The changes of four metabolic pathways in pulmonary fibrosis mice at different time periods.

Figure 3 Species difference analysis of fecal microbes.

(A) Alpha diversity analysis (Shannon diversity index) among the four groups at the species level; (B) Principal coordinate analysis (PCoA) of the fecal microbiota; (C) LDA scores for the bacterial taxa differentially abundant among Control, Model_7Day, Model_14Day, and Model_28Day.

Figure 4 Fecal microbes functional composition and difference analysis on KEGG.

(A) Functional PCoA based on the Bray–Curtis distances of fecal microbes; (B) Heatmap of TOP22 differential functional units, red represents higher abundance in corresponding samples KO, blue represents lower abundance KO; (C) The histogram of the groups KEGG primary metabolic pathways; (D) The histogram of each group KEGG secondary metabolic pathway; (E) LEfSe functional branch diagram, showing the topological relationship of the main functional groups in the sample community from KEGG pathway level 1 to level 3(from inner to outer).

Figure 5 Correlation analysis of metabolites and fecal microbes.

(A) Spearman's rank correlation between differential gut microbial species and

metabolites, 40 samples were used for Spearman's rank correlation, (B) Spearman's rank correlation between differential gut microbial functional KO and metabolites. Red, represent positive correlations; blue, represent negative correlations.

Figure 6 Graphical summary of the key results of the study.

The time-dependent changes of major serum metabolites are indicated as trend lines. The representative gut microbiome and the pathways at each time points are shown explicitly.

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TABLE LEGENDS

Table S1 Screening for differential metabolites among Model_7Day, Model_14Day, Model_28Day, and Control.

Table S2 Representative differential metabolites among Model_7Day, Model_14Day, Model_28Day, and Control.

Table S3 LEfSe (LDA effect size) analysis of all samples identified 79 taxa.

Table S4 Representative differential gut microbes functional unit in all samples.

Figures

Figure 1

a single dose of bleomycin treatment (HE and Masson staining). Representative hematoxylin and eosin stained (a, b, c, and d) and Masson's trichrome stained (e, f, g, and h) lung tissue sections are shown. The mice were sacrificed at 7 (b and f), 14 (c and g), and 28 (d and h) days after bleomycin instillation. Except for the PBS control (a and e), all panels showed lung sections from bleomycin treated mice. The lungs were inflated with 10% buffered formaldehyde. Original magnification, 5.0X; insets: 20X; (D) The results of TGF β immunohistochemistry. Positive Area Ratio, reflecting the proportion of the positive area.

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Figure 4

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